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EURL ECVAM Status Report on the Development, Validation and Regulatory Acceptance of Alternative Methods and Approaches (2015)

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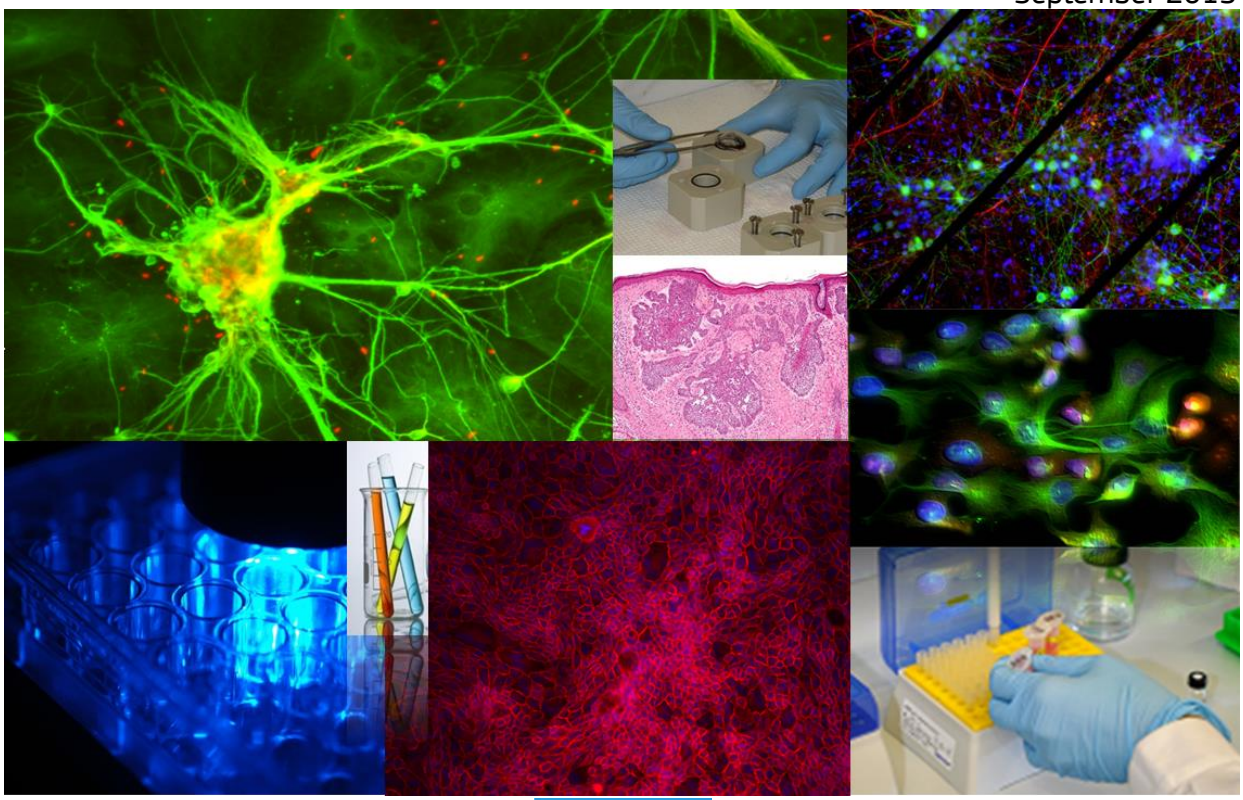

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Abstract

The EURL ECVAM status report provides an update on the progress made in the development, validation and regulatory acceptance of alternative methods and approaches and their dissemination since the last report published in June 2014. It is informing on ongoing research and development activities, validation studies, peer reviews, recommendations, strategies and regulatory/international acceptance of alternative methods and approaches and dissemination activities.
R&D activities within large European or International consortia continued in toxicity areas where 3Rs solutions are more difficult to find due to the underlying complexity of the area.
On the other hand, toxicity areas where promising non-animal approaches have been developed, their validation and regulatory acceptance/international adoption could be progressed. Particular emphasis was given to the best and most intelligent combination and integration of these different non-animal approaches to ultimately obtain the required information without resorting to animal testing.

EURL ECVAM Status Report 2015

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Executive Summary

The EURL ECVAM status report provides an update on the development, validation and regulatory acceptance of alternative methods and approaches and their dissemination since the last report published in June 2014.

All the areas in which EURL ECVAM is active are described in this report. This includes activities focusing on finding 3Rs (Replacement, Reduction and Refinement) solutions for human health-(related) effects, for environmental toxicity, for the safety and efficacy testing of vaccines, activities on knowledge-sharing and structuring, dissemination and on stakeholder engagement. For environmental effects, R&D projects aiming at improving fish toxicity testing by reducing or avoiding the use of fish are supported. Similarly projects are proposed at regulatory/international level to standardise *in vitro* methods to determine *in vitro* fish intrinsic hepatic clearance rates with an aim to enhance the reliability of Bioconcentration Factor (BCF) models, and projects which address the use of solvents in aquatic toxicity tests on fish with a view to reducing the number of fish in current OECD Test Guidelines and Guidance Documents.

Concerning human health related R&D projects, EURL ECVAM continued to be very active in the SEURAT-1 project, a major European research consortium established to evaluate the safety of chemicals considering repeated exposure in humans without using animals. Besides the cross cluster proof-of-concept activities in SEURAT-1, the individual projects (Detective, Notox, HeMiBio, COSMOS, Scr&Tox and Toxbank) yielded important milestones in the development of alternative approaches based on e.g. the development of pluripotent stem cells into specific types of cells that could be used in microbioreactors (developed within the HeMiBio project) and computer technology (explored within the COSMOS project). These results will be presented at the final SEURAT-1 symposium at the end of 2015.

In the framework of the OECD programme on the development of Adverse Outcome Pathways (AOP), EURL ECVAM has developed AOPs relevant to neurotoxicity, toxicity via disruption of the endocrine system and liver toxicity. An adverse outcome pathway is a conceptual framework constructed from knowledge that relates exposure of a type of toxic substance to subsequent molecular and cellular changes that result in illness or injury to an individual or population. This knowledge-based framework helps to guide the development of mechanistically relevant methods and models and their best integration to ultimately predict the adverse outcome of interest without the need to resort to animal tests. Most of these AOPs were introduced into the AOP Knowledge Base-Wiki, developed by JRC/EURL ECVAM in collaboration with the US Environmental Protection Agency (EPA), and are currently undergoing an OECD external review process. In addition, JRC/EURL ECVAM contributed to the development of guidance on the principles and best practices for constructing AOPs and AOP networks.

EURL ECVAM strategies in the areas of acute systemic toxicity, aquatic toxicity and bioconcentration/bioaccumulation testing as well as toxicokinetics were published in 2014 and 2015, respectively. A EURL ECVAM strategy to replace, reduce and refine the use of animals in the assessment of (developmental) neurotoxicity is currently in preparation. These strategies address the different regulatory needs in the respective fields, review the progress made on alternative approaches, identify gaps and opportunities with regard to the development and validation of alternative approaches and outline actions that should be taken to achieve an impact on the 3Rs.

Thirty test submissions were evaluated by EURL ECVAM during the period covering this report, fifteen entered via the standard EURL ECVAM test submission process and another fifteen submissions were a result of a call for submission of *in vitro* human hepatic metabolic clearance methods. For the very few promising test submissions addressing toxicity areas that are not covered by AOP or Integrated Approaches to Testing and Assessment (IATA) frameworks, EURL ECVAM consulted its network of regulators for the preliminary assessment of regulatory relevance (PARERE).

EU-NETVAL, EURL ECVAM's network of validation laboratories has been established and the validation of an Androgen Receptor Transactivation Assay (ARTA) is currently ongoing in three selected EU-NETVAL test facilities as first pilot project. The generation of experimental data using this method will support the development of an OECD performance-based test guideline and associated performance standards for ARTAs for the detection of compounds with (anti)androgenic potential. The next task of EU-NETVAL will be to assist EURL ECVAM in harmonising and standardising human hepatic metabolic clearance methods to increase the reliability of this class of *in vitro* assays to be used in regulatory decisions.

The ECVAM Scientific Advisory Committee (ESAC) peer reviewed the EURL ECVAM-coordinated validation studies on the human cell line activation test (h-CLAT), the EpiOcular™ Eye Irritation Test and the human-based CYP induction assays. In addition, it reviewed the external (to EURL ECVAM) Performance Standards-based validation study on the epiCS® test method for skin irritation testing.
EURL ECVAM Recommendations on the h-CLAT for skin sensitisation testing and on the Zebrafish Embryo Toxicity Test (ZFET) for acute aquatic toxicity testing were published. Two additional recommendations on the EpiOcular™ Eye Irritation Test and on the human-based CYP induction assays are currently being prepared.

At OECD level, EURL ECVAM is active in various programmes. Within the OECD Test Guideline programme, EURL ECVAM is leading or co-leading ten projects on the development of new test guidelines or guidance documents. Over recent years, the development of IATA has become a first priority in the search for alternative solutions to animal safety testing. In that context, EURL ECVAM is leading, within the OECD Task Force on Hazard Assessment, the development of two guidance documents (GD) that focus on

the reporting of IATA and on individual information sources used within IATA for skin sensitisation, with an aim to promote the consistent reporting of IATA within OECD member countries.

EURL ECVAM is also co-chairing with the US EPA the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics and its AOP Development Programme. Furthermore, it supports the development of consistent assessment approaches for combined exposure to chemical mixtures at international level within the OECD project on combined exposure that is led by the OECD Task Force on Hazard Assessment in collaboration with the OECD Task Force on Exposure Assessment.

At international level, EURL ECVAM is also collaborating with VICH[1] in the framework of development of VICH Guidelines on vaccines, with ICH[2] on ICH guidelines, with the WHO[3] International Programme on Chemical Safety (IPCS) and with its international partners within the International Cooperation on Alternative Test Methods (ICATM).

At EU level and in the context of REACH, EURL ECVAM continued to actively support the regulatory acceptance of alternative approaches through updates of REACH Annexes for reflecting scientific progress in the areas of skin corrosion/irritation, serious eye damage/eye irritation, skin sensitisation and acute systemic toxicity and updates of REACH Guidance on Information Requirements and Chemical Safety Assessment for skin corrosion/irritation, serious eye damage/eye irritation, respiratory irritation and for skin and respiratory sensitisation. EURL ECVAM also contributed to a series of free webinars focusing on alternative methods and testing strategies that can be used to meet REACH requirements, organised by the PETA International Science Consortium, Ltd., and Chemical Watch.

EURL ECVAM's dissemination activities range from well-established EURL ECVAM databases such as the EURL ECVAM's DataBase service on ALternative Methods to animal experimentation (DB-ALM), the QSAR Model Database and the EURL ECVAM Search Guide to the revision of the Tracking System for Alternative Test Methods towards Regulatory acceptance (TSAR), the chemical information portal ChemAgora, the Reference Chemicals List for Validating Alternative Methods CheList and the EURL ECVAM Genotoxicity and Carcinogenicity Database of Ames Positive Chemicals.

EURL ECVAM furthermore increased its engagement with regulators from the EU Member States and EU agencies (ECHA[4], EMA[5], EFSA[6]) via PARERE; with all EU industry associations

[1] International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

[2] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

[3] World Health Organisation

[4] European Chemicals Agency

[5] European Medicines Agency

dealing with chemicals, pharmaceuticals and cosmetics, non-governmental organisations and academia via its ECVAM Stakeholder Forum (ESTAF) and the EPAA[7], and the 3Rs community as a whole, for a sustained investment, commitment and support of all actors in the field of alternative approaches.

[6] European Food Safety Authority

[7] European Partnership for Alternative Approaches to animal testing

1. Introduction

The EURL ECVAM status report 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 in June 2014. It covers the period May 2014 to September 2015 and reports on research and development, and validation activities as well as on activities which promote the regulatory acceptance and use of alternative approaches and their dissemination. It is intended to inform interested parties and serve multiple purposes, including 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.

2. Research and Development Activities on Alternative Methods

2.1. SEURAT-1: 2015 - the final year of the largest European project on alternative methods

SEURAT-1 is a major European research consortium established to evaluate the safety of chemicals considering repeated exposure in humans without using animals. It is a public-private partnership co-financed by the European Commission's FP7 Health Programme and Cosmetics Europe. SEURAT-1 consists of six individual research projects and one coordination action (COACH), and combines the research efforts of over 70 European universities, public research institutes and companies.

The SEURAT vision is to fundamentally change the way we assess the safety of chemicals, by superseding traditional animal experiments with a predictive toxicology that is based on a comprehensive understanding of how chemicals can cause adverse effects in humans. The SEURAT strategy is to adopt a toxicological mode-of-action framework to describe how any substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental (*in vitro*) models that predict quantitative points of departure needed for safety assessment. One of the SEURAT-1 objectives is to demonstrate a multiple level proof-of-concept (theoretical, methodological, and application) for repeated dose systemic toxicity, but in principle also applicable to other endpoints.

Firstly, a theoretical Adverse Outcome Pathway (AOP) describing the key events of the biological process initiated by a chemical stressor is developed (Level 1). Secondly, a testing strategy for toxicity prediction, based on AOP knowledge relevant to the toxicity to be predicted is needed (Level 2). Typically a combination of *in vitro*, *in silico* and *in chemico* data is required to trigger selected key events. Finally, the results of testing strategies in combination with already existing data (physical chemical information, animal or human *in vivo* data or other) and biokinetic modelling, could provide sufficient evidence to support chemical safety assessment (Level 3).

For Level 1, several AOPs were developed within SEURAT-1, primarily with relevance to hepatotoxicity. The pathway description from protein alkylation to liver fibrosis has been revised, extended, and modified. In October 2014, an expert workshop was organised to discuss and analyse the AOP on liver fibrosis, and to identify and prioritise data gaps for further research. Collaboration for the confirmation and elucidation was agreed and further studies for measuring relevant key events (KEs) and possibly their quantitative relationships to up- and down-stream KEs were discussed.

The updated AOP has gone through internal OECD review and will be submitted to another review by external experts.

The applicability of this AOP to liver fibrosis for Nanoparticles has been investigated and described. A scientific paper titled "The Adverse Outcome Pathway approach in nanotoxicology" has been submitted for publication.

On Level 2, there are currently eight case studies with different toxicity prediction goals that are close to finalisation. On Level 3, there are three case studies, for which chemicals safety assessments are carried out based on (1) an *ab initio* approach (no animal data available on the chemical itself or similar chemicals), (2) read-across from data-rich source substances to the substance to be assessed using strengthening arguments from alternative data, and (3) accepting a Threshold of Toxicological Concern (TTC) to be good enough based on improved substance characterisation using computational models. All three case studies apply a conceptual framework for chemicals safety assessment developed within SEURAT-1.

Besides the cross cluster proof-of-concept activities, the individual projects are finalising their deliverables. At the SEURAT-1 fifth annual meeting in Barcelona on 21-22 January 2015, the project coordinators reported major achievements and progress since the last annual meeting and explained how to successfully conclude the activities by the end of 2015. DETECTIVE reported identification of novel biomarkers for kidney, liver and heart. They also showed that differentiated human-skin derived cells can acquire hepatic properties, e.g. they demonstrate steatotic functions when exposed to a chemical known to cause fatty liver, and therefore they are suitable to use for *in vitro* hepatotoxicity testing. All cell systems tested within DETECTIVE were found to have a toxicological memory in their genetic profile after exposure to chemical stressors. NOTOX demonstrated the results of a multi-scale model for steatosis prediction including repeated dose 3D *in vitro* models, using HepaRG, in combination with computational biokinetic modelling mimicking human exposure. The COSMOS DataBase, with a record of more than 80.000 cosmetic relevant substances, is now publicly available and is further complemented with a TTC (Threshold for Toxicological Concerns) database, 'COSMOS space' with representations of the chemical space of cosmetic ingredients and easy-to-use biokinetic models in the open source software KNIME (Konstanz Information Miner). COSMOS also developed several profilers that can be applied to characterise chemicals, for example binding potential to specific proteins or skin penetration coefficients. SCR&Tox has finalised protocols for

differentiations of human pluripotent stem cells into keratinocytes, neurones, cardiocytes and hepatocytes, and in addition established technologies for large scale production and banking. HeMiBio12 presented results from a unique model predicting fibrosis composed of 3D co-cultured liver cell lines in a flow over bioreactor with implemented electronic sensors for detection of read-outs. ToxBank13 reported good progress in collecting resulting data from the SEURAT-1 projects as well as the proof-of-concept case studies.

The main achievement of each project and the results of the proof-of-concept case studies will be presented at the final SEURAT-1 symposium planned in Brussels on the 4th of December 2015.

2.1.1. Cosmetics Europe Contract

A collaborative arrangement between the JRC and Cosmetics Europe aims to supplement the work programme of the SEURAT-1 cluster. This research initiative, although motivated by the Cosmetic industry, is also relevant to other industrial sectors (e.g. industrial chemicals, pharmaceuticals).

The aims of this project are (a) to facilitate cluster interaction and to demonstrate proof-of-concept at three different levels, namely 1) defining mode-of-action (MoA), 2) predicting toxicity, and 3) supporting safety assessment decisions, to supplement the computational modelling activities of the cluster and to generate large reference datasets to support the establishment of toxicological MoA. In brief, the following work was carried out by EURL ECVAM:

- A case study was designed to predict repeated dose liver toxicity by developing a MoA-based classification model that was aimed at distinguishing between hepatotoxicants and non-hepatotoxicants. Hepatotoxicity was mainly related to three major liver adverse outcomes associated with repeated dose exposure: cholestasis, fibrosis and steatosis. Concentration-response data sets for 90 reference chemicals relevant to the three hepatotoxicity MoA mentioned above were generated using a well characterised *in vitro* liver system (HepaRG cells). A repeated dose exposure scenario and high-throughput screening (using a 96-well plate format) was employed. Mitochondrial damage, reactive oxygen species formation (indicator of oxidative stress), formation of neutral lipid droplets (indicator of chemically induced steatosis), apoptosis and cell count (indicator of cell viability), were the selected key events studied. The read-outs were performed using high content imaging. The lowest concentration at which the effect was observed (i.e. the first significant effect level *in vitro*) was determined. The project showed that the MoA knowledge is fundamental to define the prediction goal of a testing strategy, to support the identification of the biological events to be measured, to select the most suitable *in vitro* cell model and to guide the entire design of the study by improving the selection of chemicals, the definition of exposure protocol and the strategy for data analysis.

- A human Physiologically Based Kinetic (PBK) model that simulates the *in vivo* kinetics of caffeine was developed and coupled with a Physiologically Based Dynamic (PBD) model that simulated the dynamics of caffeine-induced effects on liver cells. The PBD model used elements of our previously developed Virtual Cell based (VCB) model, which simulates the fate and effects of chemicals in cell cultures (Zaldivar *et al.*, 2010, 2011, 2012). The resulting PBK/D modelling was implemented in the KNIME workflow and several consumer exposure scenarios, based on the expected use of three caffeine-containing cosmetic products, were explored. This project showed how *in silico* models describing the kinetics and dynamics of chemicals in cell cultures and the human body can be applied in risk assessment to translate *in vitro* toxicity data into predictions of human toxicity under defined consumer exposure scenarios (*in vitro* to *in vivo* extrapolation; IVIVE) (Gajewska *et al.*, 2015).
- Different modelling approaches, including physiologically-based toxicokinetics (PBTK) and quantitative structure-activity (QSAR) modelling were used to illustrate how they could be employed in the risk assessment process for cosmetic substances. The various theoretical models were built, applied, and integrated in order to develop an approach for assessing systemic exposure or internal dose (dermal bioavailability including metabolism) upon topical (dermal) use of cosmetic products and to investigate whether dermal threshold values could be set for humans. The work focused on two cosmetics-relevant chemicals, coumarin and caffeine, for which dermal and oral repeat dose toxicity data were available. The results showed that assessment of dermal bioavailability, expressed as area under the curve (AUC) and using integration of PBTK modelling for whole body kinetics and QSAR modelling for dermal penetration is possible, albeit with unknown uncertainties linked to assumptions in the models. In addition, extrapolation from external oral to dermal threshold values is practically impossible and, consequently, not a generally feasible approach. Differences between various human dermal exposure scenarios can have significant effects on the qualitative aspects of the concentration-time curve (the form) as well as on the more quantitative aspects of the curve, expressed as AUC and C_{max}. Therefore, it is more realistic and feasible to compare the effects of oral and dermal exposure scenarios by using internal threshold values and an internal margin of safety approach (Gajewska *et al.*, 2014).

2.1.2. DETECTIVE (Detection of endpoints and biomarkers of repeated dose toxicity using *in vitro* systems)

The DETECTIVE project[8] aims at the identification and evaluation of *in vitro* biomarkers for repeated dose toxicity testing. To obtain these biomarkers, human cell models representative of the liver, kidney and heart are exposed to model compounds, as selected by the golden compound working group of SEURAT-1[9]. The effects are then assessed at

[8] http://www.detect-iv-e.eu

[9] http://www.toxbank.net/compound-wiki

the –omics (which deliver data on the entire cellular situation at the molecular level) and the functional levels (which give more insight into the effects of toxicants on specific cell functions of interest). The project is also exploring whether repeated dose effects on epigenetics and microRNA (miRNA) expression expand our understanding of toxic modes of action. Relevant, specific, sensitive and predictive biomarkers will be selected based on integrative statistical analysis, systematic verification and correlation with *in vivo* data. A comprehensive overview of the results obtained until now in the project can be found in the Annual reports of the SEURAT-1 Research Initiative[10] (SEURAT-1, 2011; 2012; 2013; 2014). Some highlights from the project include:

- The establishment of an adverse outcome pathway framework for drug-induced cholestasis (Vinken *et al.*, 2013).
- The development of a panel of fluorescent reporter cell lines that allows the quantitative assessment of cellular stress responses in cells after chemical exposure in relation to organellar perturbations (Wink *et al.*, 2014).
- A publicly available toxicotranscriptomics directory[11] which provides information for all genes whether they are up- or down-regulated by chemicals and, if yes, by which compounds. The directory, which also contains information about stereotypical stress response, liver disease-associated genes, unstable baseline genes, and biological function, offers a basis for a rational choice of candidate genes for biomarker evaluation studies and represents an easy to use source of background information on chemically influenced genes (Grinberg *et al.*, 2014).

2.1.3. SCR&Tox (Stem Cells for Relevant Efficient Extended and Normalized Toxicology)

The Scr&Tox project developed a variety of test systems derived from human pluripotent stem cell lines as they may be expanded indefinitely and triggered to differentiate into any cell type. The protocols for cell differentiation of human embryonic and induced pluripotent stem cells (iPSCs) towards 5 different lineages (liver, heart, CNS, epidermis and muscles) have been established and applied to *in vitro* toxicity testing using a variety of functional cell specific assays. Recently, the genetically engineered induced pluripotent stem cell line expressing a reporter for activity of the Nrf2 transcription factor, as a marker of cell responses to oxidants, was created. This cell line will be differentiated into keratinocytes (2D and 3D epidermis) and neural cells to evaluate the relevance of Nrf2 activation as a general “toxicity” pathway for toxicity testing.

[10] http://www.seurat-1.eu

[11] http://wiki.toxbank.net/toxicogenomics-map/

2.1.4. COSMOS (Integrated *in silico* models for the prediction of human repeated dose toxicity of COSMetics to Optimise Safety) Project

2.1.4.1. COSMOS Project Background

EURL ECVAM is a partner in the COSMOS project[12] which is developing publicly available computational workflows based on the integrated use of open-access and open-source models for the prediction of repeated dose toxicity (Anzali *et al.*, 2012). The work includes: a) the establishment of an inventory of cosmetic substances (including identifiers and chemical structures) and a repeat dose toxicity database (including oral and dermal data); b) the development of novel ways of establishing thresholds of toxicological concern (TTC); c) the development of innovative toxicity prediction strategies based on molecular and QSAR modelling; d) the development of a multi-scale modelling approach to predict target organ concentrations and to extrapolate from *in vitro* to *in vivo* exposure scenarios; and e) the integration of databases and modelling tools to provide publicly accessible computational workflows for use in the safety assessment of cosmetics.

2.1.4.2 COSMOS Database

The COSMOS database is a freely accessible comprehensive and reliable resource for repeated dose toxicity data, including subacute, subchronic, and chronic studies as well as carcinogenicity studies (Rathman *et al.*, 2013). It is also linked to high quality and validated chemical structures to facilitate modelling. A COSMOS webinar entitled "COSMOS DB: A New Database of Toxicological Information to Support Knowledge Discovery", hosted by the American Society for Cellular and Computational Toxicology (ASCCT), is accessible from Youtube[13]:

2.1.4.3 Threshold of Toxicological Concern

The Threshold of Toxicological Concern (TTC) work was carried out with the support of two International Life Sciences Institute (ILSI) (Europe) expert groups. The main developments were the establishment of a COSMOS TTC dataset containing peer-reviewed NO(A)EL values, and the development of the oral-to-dermal extrapolation approach to investigate whether the TTC approach can be applied to dermal exposure scenarios. This work was presented in a webinar entitled "Threshold of Toxicological Concern – an approach for safety assessment and its applicability to cosmetics-related chemicals" on 24 July 2014. The webinar is accessible from the ASCCT[14]:

2.1.4.4. Modelling of Key Events Leading to Liver Steatosis

Several computational approaches have been applied and integrated to develop an *in silico* strategy for the evaluation of potential activation of nuclear receptors (in particular, the Liver X Receptor [LXR] and the Peroxisome Proliferator-Activated Receptor γ [PPARγ] involved in the development of liver steatosis). These methodologies include: molecular

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

[13] https://www.youtube.com/watch?v=oLD0mHxNz6c&feature=youtu.be

[14] http://www.ascctox.org/meetings.cfm

modelling, QSAR (quantitative structure-activity relationship) and structural alerts. The use of molecular modelling approaches as a means of supporting the AOP framework for toxicity prediction is one of the Level 2 SEURAT-1 case studies (Al Sharif *et al.*, 2014; Tsakovska *et al.*, 2014).

2.1.4.5 Toxicokinetic Modelling

Physiologically-based kinetic (PBK) models can be 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 processes of absorption, distribution, metabolism and excretion (*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. During 2014-2015, PBK models have been developed for selected compounds (Gajewska *et al.*, 2014a, 2014b, 2015). 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 extended to include a mitochondrial compartment. Furthermore, VCBA models were made publicly accessible by implementation as KNIME workflows. In a multi-scale modelling approach, PBK models can be coupled with VCBA models to enable realistic estimates of *in vivo* effects from *in vitro* toxicity data. This work was presented on 13 April 2015 during an ASCCT-hosted webinar entitled "Automated *in silico* tools for *in vitro* to *in vivo* extrapolation". The webinar is accessible from Youtube[15]:

2.1.4.6 Computational Tools

A number of KNIME workflows have been developed to support *in silico* modelling, including: i) analysis of chemical space using Principal Component Analysis (PCA) on molecular descriptors and functional groups; ii) evaluation of nuclear receptor binding potential, based on structural alerts and QSAR approaches; iii) prediction of skin and gastrointestinal absorption; and iv) biokinetic modelling (VCBA and PBK models for selected chemicals).

These computational workflows can be accessed via the KNIME WebPortal[16], following registration through COSMOS Space[17]. COSMOS Space is a platform for the sharing of predictive toxicology resources (e.g. data sets, models, workflows, documentation). In addition to linking with KNIME workflows developed by the COSMOS project, COSMOS Space also provides a link to the COSMOS database.

[15] https://www.youtube.com/watch?v=vJc1UEbuWdE&feature=youtu.be

[16] (http://knimewebportal.cosmostox.eu)

[17] http://cosmosspace.cosmostox.eu)

2.2. CALEIDOS Project

EURL ECVAM played an advisory role in the CALEIDOS (Chemical Assessment according to Legislation Enhancing the *In silico* Documentation and Safe use) project[18]. This was a DG Environment-funded project under the Life+ programme (January 2013-June 2015). The project explored the regulatory applicability of non-testing methods (QSAR and read-across) to REACH substances. Using data on REACH-registered chemicals, the predictive performances of QSAR models (mostly freely available, but also some commercial models) were evaluated and compared for the following endpoints: Ames mutagenicity, rodent carcinogenicity, developmental toxicity (rats and rabbits), logP (relevant for waiving of bioaccumulation testing and the screening level assessment of bioaccumulation), and bioconcentration in fish (BCF).

The project has also provided a freely available software tool called Toxread which currently facilitates read-across for mutagenicity and BCF predictions.

2.3. Fish Toxicity R&D Projects

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

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

As a follow-up of the CEllSens[19] 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 EAWAG (K. Schirmer; CEFIC LRI project ECO8.3-NC3Rs-EAWAG[20]). Results should become available during 2015.
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).

2.3.2. 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; OECD 2013a). A CEFIC LRI-funded project (LRI-ECO20-UA[21]) aims 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. The report of a recent ILSI Health and Environmental Sciences Institute (HESI) workshop (Villeneuve *et al.*, 2014) discusses AOPs relevant to FELS toxicity and how they could be discovered and annotated.

[18] http://www.caleidos-life.eu/

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

[20] 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/

[21] 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/

At a recent AOP workshop[22], co-organised and co-sponsored by the JRC, AOPs related to fish chronic toxicity with a focus on fish growth were discussed and the outcome was published (Groh *et al.*, 2015a; Groh *et al.*, 2015b).

2.3.3. 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 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 (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 related to developing and applying useful ecotoxicological threshold of concern (eco-TTC) concepts. EURL ECVAM is contributing to this initiative.

2.4. Activities within EPAA

The European Partnership for Alternative Approaches to Animal Testing[23] (EPAA) is a collaboration between the European Commission, European trade associations and companies from seven industry sectors.

The partners are committed to pooling knowledge and resources to accelerate the development, validation and acceptance of alternative approaches to animal use in regulatory testing. The overall aim is the replacement, reduction and refinement (3Rs) of animal use in regulatory testing. JRC, represented by EURL ECVAM, is one of the Commission services that are members of the EPAA.

2.4.1. Stem Cells

The development of alternatives to replace animal-based safety testing requires the development of suitable *in vitro* systems possessing physiological relevance. Induced Pluripotent Stem Cells (iPSC), derived from human adult tissues, can be manipulated to produce all cell types and offer great potential as a pivotal alternative to animal use in safety testing. The development of iPSC and establishment of physiological relevance in safety testing requires a multidisciplinary, international approach to combine the expertise in academic, industry and regulatory groups.

In 2014, the stem cells working group of EPAA together with collaborating groups and building on previous workshops and expert meetings, developed the 'Research Prospectus',

[22] https://aopkb.org/saop/workshops/somma.html

[23] The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a public-private collaboration; see also: http://ec.europa.eu/growth/sectors/chemicals/epaa/index_en.htm

a document which defines specific research needs and priorities for the development of iPSC as a central and accepted test system for alternative methods in safety assessments. It also proposes possible topics for calls under the Horizon 2020 Work Programme. The document was discussed with and welcomed by the European Commission (DG RTD).

In succession of the first *International Stem Cells Forum* that was initiated by EPAA in 2013, a second *Forum* took place in September 2014, jointly organized by EPAA and the British Centre for Drug Safety Science (CDSS[24]). The event gathered 25 invited experts from Europe and US and focused on benchmarking of stem cell assays in safety assessment across international consortia. Hepatotoxicity was at the forefront of discussions. Participants from industry, academia, regulators (US National Institutes of Health, European Medicines Agency), EURL ECVAM and civil society agreed that reinforced collaboration at international level could accelerate the development of reliable stem-cells based alternative methods. A paper summarising the conclusions of the event was published in the peer-reviewed journal *Stem Cells and Development* (Suter-Dick L *et al*., 2015).

2.4.2. Toxicokinetics - Exposure Prediction tool

A new project funded by EPAA started in June 2014 which aims at the further development of an existing bioinformatics tool to convert external exposure data to internal exposure in humans and vice versa. The tool uses human absorption, distribution, metabolism and excretion (ADME) characteristics that may have been obtained using QSARs as well as *in vitro* approaches. The outcome of the project will be an open source web application programmed in R syntax[25]. It will serve the needs of *in vitro* as well as *in vivo* toxicologists, risk assessors and mathematical modellers for the purpose of quantitative *in vitro* to *in vivo* as well as *in vivo* to *in vitro* dose metric extrapolation. It is based on a physiologically based toxicokinetic (PBTK) model structure. Once finalised, the tool will not only be freely accessible in all its modules, but also downloadable to the user's PC. This avoids the need for uploading proprietary information to the web and thus assures the confidentiality of the user's data.

The beta-release of the tool is expected for the end of 2016 and a second project phase, which will generate the final open-access version, is under consideration at EPAA. EURL ECVAM participates in the technical advisory team to this project.

2.4.3. EPAA Science Awards/Prizes

The EPAA Awards are granted to young scientists (3Rs Science Prize) or laboratory technicians (3Rs Technician Prize) whose work has brought an outstanding contribution to

[24] https://www.liv.ac.uk/drug-safety/

[25] https://www.r-project.org/

the development and implementation of alternatives to animal testing. Both Science and Laboratory technician prizes are awarded biannually.

In 2014, the EPAA granted the 3Rs Science Prize to a scientist from the University of Tampere/FICAM for her outstanding research that helped to develop a novel *in vitro* human-based vascular network model. By making use of cell co-cultures, the model is meant to be used for angio-/vasculogenesis testing, resulting in a tissue-like system. The Prize winner presented her work to the participants of the EPAA Annual Conference in November 2014.

3. Test Method Submissions

In total, 13 test submissions were sent to EURL ECVAM for evaluation during the period covered by this report, of which eight were pre-submissions, reporting information in the Test Pre-Submission Form (TPF), and five were full submissions, reporting information in the Test Submission Template (TST), usually complemented by annexes. In addition, two full submissions that had been received in late 2013, i.e. the Yeast Androgen Screening Test (YAS) and the Ocular Irritection Test were also reviewed by EURL ECVAM in 2014. Thus, in total, EURL ECVAM assessed 15 test submissions during this period.

Noteworthy, all seven full submissions were reporting information and data on external validation studies submitted to EURL ECVAM in view of peer review by the ECVAM Scientific Advisory Committee (ESAC) and an EURL ECVAM recommendation.

For each test submission, an assessment report was prepared, an assessment meeting was held and reply letters were drafted according to the internal JRC quality procedure.

Besides the assessment of the test submissions received, EURL ECVAM also reviewed a validation project plan of a test that had been submitted in 2011 (SENS-IS) and further to the definition of the EURL ECVAM strategy on toxicokinetics, also replied for a second time to a test method developer of a skin penetration method submitted in 2012 (Skin PAMPA). In addition, EURL ECVAM consulted its network of regulators PARERE (Preliminary Assessment of Regulatory Relevance Network) on the regulatory relevance of a test method that had been initially submitted in 2009 (GreenScreen HC) and for which an updated full submission was received in 2014 and launched the PARERE consultation process on a test method for teratogenicity testing (devTox qp).

In addition to the standard EURL ECVAM test submission process via the EURL ECVAM website, EURL ECVAM also launched a call for *in vitro* human hepatic metabolic clearance methods (HHMC) in March 2014 that resulted in the submission of 15 HHMC methods.

3.1. Test Method Submission Related to Acute Fish Toxicity

RTgill-W1 test method

EURL ECVAM assessed the pre-submission on the RTgill-W1 cell line assay received in March 2014. The method uses the fish cell line RTgill-W1 derived from rainbow trout gills (Tanneberger *et al.*, 2013). Cells are exposed for 24 h to a series of test chemical concentrations. Cytotoxic effects (EC50 values) are determined by measuring the cell viability using three fluorescent dyes (AlamarBlue for cellular metabolic activity, 5-carboxyfluorescein diacetate acetoxymethyl ester for cell membrane integrity and Neutral Red for lysosomal membrane integrity testing). Cell viability of the exposed cells is expressed in % of the control cells. The lowest EC50 values are used for predicting acute fish toxicity. Based on the outcome of the assessment, EURL ECVAM invited the test submitter to provide a full test submission as soon as more data on the within and between laboratory reproducibility become available (see 2.3.1).

3.2. Test Method Submissions Related to Genotoxicity

3.2.1. Green Screen HCTM (GSHC) Assay

A full test submission was received in May 2013 on a method intended to predict the *in vivo* genotoxic potential of chemicals: the Green Screen HC™ (GSHC) assay. The GSHC is a microplate format genotoxicity plus cytotoxicity screening assay which uses the DNA damage-inducible "Growth Arrest and DNA Damage 45 alpha" (*GADD45a*) - Green Fluorescent Protein (GFP) reporter gene, expressed in the p53-competent human lymphoblastoid TK6 cell line (Hastwell *et al.*, 2006; Jagger *et al.*, 2009).

Upon treatment with a DNA damaging agent, *GADD45a* is highly induced, mostly in a p53-dependent manner. When *GADD45a* transcription is increased over its constitutive level, cells accumulate GFP, the fluorescence of which can be measured as a proportional assessment of genome damage and genotoxic stress.

This test method underwent an external (non EURL ECVAM-coordinated) validation study and all available information/data were submitted to EURL ECVAM for a retrospective validation in view of ESAC peer review. Based on the information provided, the method appeared to be mechanistically and biologically relevant in relation to genotoxicity. A standardised protocol is publicly available in the EURL ECVAM DB-ALM since July 2012 (http://ecvam-dbalm.jrc.ec.europa.eu/). The data provided in this submission were convincing enough to support the use of the test method as a screening tool in early phases of compound discovery and development. However, due to the properties of the method as a general indicator of genotoxicity the actual role of the GreenScreen HC™ assay within current regulatory testing approaches needed to be further elucidated. In light of this, and before considering peer review by ESAC, in June 2014, EURL ECVAM launched a consultation with the PARERE (Preliminary Assessment of Regulatory Relevance) network of regulators. EURL ECVAM considered a number of scenarios, some of which might have had an added value to current genotoxicity testing practices. Scenario 1:

GreenScreen as a substitute to the Ames test in ICH S2 (R1) Option 2; Scenario 2: GreenScreen as an alternative to the *in vitro* battery; Scenario 3: GreenScreen as an alternative to one of the currently used *in vitro* tests; Scenario 4: GreenScreen as an additional test to the current *in vitro* battery. PARERE members were asked to give feedback on the following matters: relevance of the method for regulatory testing of genotoxicity, relevance in respect of different sectors, evaluation of the four different scenarios, and other scenarios in which the use of such a method could be envisaged.

In agreement with the main conclusion drafted by EURL ECVAM in the Assessment Report on the Full Test Submission, the GreenScreen HC™ assay has been considered by PARERE as a suitable method to identify the genotoxic potential of substances, e.g. as a screening tool at early phase of drug discovery and development. However, the impossibility of deriving specific information on the mechanism(s) leading to the observed genotoxic effects (endpoints: mutations, structural and numerical chromosomal aberrations) upon treatment with any type of substances appears to be one of the main reasons for questioning its regulatory relevance.

PARERE highlighted that the lack of this endpoint information could lead to an increase of *in vivo* experiments instead because of the lack of guidance on the specific test to be performed in animals when needed/required. However, the GreenScreen HC™ assay was considered to be potentially useful as an additional test in a weight of evidence strategy to follow up, on a case-by-case basis, and to clarify the relevance of positive results from the standard *in vitro* battery. In the future, the GreenScreen HC™ assay could make an important contribution to satisfying regulatory information requirements across many sectors, if it was incorporated into a suitable Integrated Approach to Testing and Assessment (IATA) for genotoxicity. The assay may reflect a mechanistic key event in a possible Adverse Outcome Pathway for carcinogenicity and, as such, it could be considered useful to provide sensitive dose-response information that might be meaningful within an IATA aimed at estimating point(s) of departure (e.g. no-effect level) useful for risk assessment.

3.2.2. γH2AX In cell western (ICW) test method

A test pre-submission on a new *in vitro* method intended for testing genotoxicity, the γH2Ax in Cell western (ICW) assay, was received in late May 2014. The assay was presented as a 96-well plate format capable of detecting simultaneously the cytotoxic and genotoxic potential of compounds in different proliferating and metabolic competent cell lines (human or rodent).

The test aims at assessing γH2AX histone phosphorylation as a consequence of global genotoxic insult. The method relies on the strong correlation between the γH2AX histone and DNA damage, DNA double strand breaks (DSBs), and the γH2AX histone recognition as a suitable pre-cancerous biomarker *in vivo*. The test uses the 'in cell western technique' which is an immunohistochemistry *in situ* (in the well) test coupled with infra-red

fluorescence detection, determining the change in phosphorylation of γH2AX histone levels (genotoxicity) and DNA content (cytotoxicity) compared to control cells.

Based on the information provided, EURL ECVAM considered the method biologically and mechanistically relevant in relation to genotoxicity. However, the assay , in its current form, needs further development with regard to method definition, optimisation and standardisation (accuracy values, etc.) before entering into a formal validation process. Moreover, its impact on the 3Rs, its role within the current regulatory context and its applicability domain need to be further explored. A final response was delivered to the submitter in October 2014.

3.3. Test Method Submission Aiming to Address Various Endpoints

Light Up Cell System (LUCS)

The pre-submission of the Light Up Cell System (LUCS) was received in 2014. It was submitted to EURL ECVAM to replace and close a previous submission received in 2011, the Dequenching After Photobleaching (DAP) assay. The submitter claimed that the LUCS would be able to address acute toxicity and genotoxicity, to replace 3T3 NRU in its applications, including phototoxicity, and to be applied for skin corrosion and irritation. The submitter based this assertion on the possibility to use the assay in any cell types, from prokaryotes (e.g. *Escherichia coli*) to eukaryotes (e.g. human, plant or fish cells), given that the cell type used would determine the potential application of the assay. The LUCS test method measures nucleic acid alteration (e.g. DNA damage) in living cells through fluorescence readout following their treatment with a toxic substance. The underlying mechanism is the alteration of nucleic acids (DNA and/or RNA) by Reactive Oxygen Species (ROS) which trigger the observed variations of fluorescence in the cells.

- For the human acute oral systemic toxicity, the performance of the assay was not evident from the data shown. Additionally, it was not obvious what advantages the LUCS offered in comparison to other *in vitro* assays, e.g. the 3T3 neutral red uptake (3T3 NRU) cytotoxicity assay for predicting official acute oral toxicity categories.
- For the genotoxicity area, the LUCS has limited applicability as it does not directly measure damage to DNA or RNA, because ROS formation is only one of the mechanisms involved in DNA damage. The assay does not provide information on the extent of the damage either. The induction of permanent transmissible changes in the amount or structure of the genetic material is a regulatory requirement across sectors, which is not addressed by the LUCS. The test method was therefore not considered to be tailored for the genotoxicity assessment.
- Finally, there was not enough evidence to judge the relevance of the test method for its potential applications in phototoxicity or skin irritation/corrosion.

Overall, the test method was not prioritised since the intended purpose (effect of interest), applications and its possible regulatory relevance were unclear.

3.4. Test Method Submission Related to Respiratory Permeability/Penetration (Toxicokinetics)

MucilAir™

A pre-submission on an *in vitro* cell model of the human airway epithelium for the trans-epithelial permeability assessment (MucilAir™) was received in 2014. This method provides Papp (apparent permeability) values for the upper airways. Some shortcomings were identified during the evaluation process. For instance, it was not evident which human model (bronchial or nasal) was used to generate the results presented and which model would be preferred in specific circumstances. Furthermore, clear purpose and scope for the use (regulatory or non-regulatory, quantitative for PBTK modelling or categorisation when used within testing strategies) was lacking. Various validation approaches for assessing the reliability and relevance of *in vitro* ADME/TK methods are under consideration at EURL ECVAM and described in the EURL ECVAM Strategy for Achieving 3Rs Impact in the Assessment of Toxicokinetics and Systemic Toxicity[26]. In addition to the current development of standards in the area of metabolic clearance and stability (see 3.10), EURL ECVAM intends to address the area of absorption which will involve, among other tasks, the identification of representative methods which could be used to support the development of standards. In the light of all these facts the test method did not progress at that stage but may be reconsidered in the wider context of ADME/toxicokinetics and development of standards.

3.5. Test method submission related to Endocrine Disruption

Yeast Androgen Screening (YAS) test

The YAS assay is a screening assay for measuring endocrine disruption. It uses yeast cells that are transformed with a human androgen receptor and a *lacZ* reporter gene, and, measures the response towards chemicals with (anti) androgenic potential. The method was submitted to EURL ECVAM at the end of 2013 to be considered for an ESAC peer review. A full validation study had been carried out with 4 laboratories. The submission dossier was assessed by EURL ECVAM in early 2014 and feedback was requested from the submitter for some critical procedural aspects prior to continuing the evaluation. This assay may have potential to be annexed to a future Performance Based Test Guideline (PBTG) for Androgen Receptor Transactivation Assays (ARTAs).

3.6. Test Method Submission Related to Teratogenicity

DevTox ^{qP}

EURL ECVAM has received a pre-submission on a teratogenicity test method in September 2014. The assay is based on the measurement of changes in the ornithine/cystine ratio (metabolic biomarkers) present in the media after exposure of undifferentiated human induced pluripotent stem (iPS) cells to a chemical. Based on the obtained results, the test

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

developer suggests that any perturbation in this metabolite ratio past the established threshold will have the potential to cause developmental toxicity. The submitter claims that these two biomarkers provide predictive information on teratogenicity in humans.

The assay is currently used to assess developmental toxicity risk potential in discovery and preclinical development programs for prioritisation of the development of new compounds using a human embryonic stem cell version of the assay (not iPS cells). Further to EURL ECVAM's assessment, the submitter provided clarifications on some critical issues. However, since the usefulness of the test method in the current regulatory context regarding developmental toxicity mediated by teratogenicity remains unclear, EURL ECVAM launched a consultation with its network of regulators (PARERE) which is currently on-going.

3.7. Test Method Submissions Related to Skin Sensitisation

3.7.1. The Genomic Allergen Detection (GARD) test method

The Genomic Allergen Detection (GARD) test method (Johansson *et al.*, 2011, 2013), a transcriptomics-based *in vitro* assay proposed to discriminate between skin sensitising and non-sensitising chemicals on the basis of the expression level of mRNA transcripts for a panel of 200 genes in MUTZ-3 cells, was resubmitted to EURL ECVAM in the first quarter of 2015. Since the initial submission in 2013, the test method has undergone additional refinement including modifications to the prediction model. Following the evaluation of the test submission EURL ECVAM concluded that, although the test method appears to be of relevance for the assessment of the skin sensitisation potential of substances, additional data need to be generated with the latest versions of the protocol and the prediction model before the test method can be considered for progression into the next phases of the validation process.

3.7.2. U-SENS™ (former MUSST)

The U-SENS™ (former MUSST) which distinguishes sensitisers from non-sensitisers on the basis of CD86 membrane protein enhanced expression in U937 cells, includes an addition to the prediction model, consisting of a score derived from six rules that allows classifying a chemical, which would have been considered inconclusive on the basis of the original prediction model, as a sensitiser or non-sensitiser (Piroird *et al.*, 2015). The U-SENS™ assay underwent an external validation study conducted in two phases. The results of the first phase were submitted to EURL ECVAM in 2013 and were judged to be insufficient to enter the EURL ECVAM peer review process at that time. A revised submission was received in the last quarter of 2014. This second submission is complemented with additional results generated during the second phase of the validation study. The evaluation of the full submission performed by EURL ECVAM indicated that the information provided in the submission would qualify the test method to enter the peer-review process upon the clarification of a few aspects of the submission by the test developer.

3.7.3. SENS-IS

The SENS-IS assay (Cottrez *et al*., 2015) is a gene expression-based test method proposed to discriminate between sensitisers, non-sensitisers and irritants by analysing the expression of a panel of 65 genes grouped in one gene set for irritancy (IRR) and two (SENS-IS and ARE) for sensitisation. A test substance is classified as sensitiser on the basis of the number of overexpressed genes (compared to solvent control) measured by qRT-PCR in Episkin tissues (SkinEthic, France). In addition, the test method allows the classification of sensitisers into potency categories on the basis of the concentration of chemical needed to induce a positive response. Subsequent to the submission of the SENS-IS method to EURL ECVAM, the test submitter communicated the intention to undertake an external validation study on the method. During the first quarter of 2014, EURL ECVAM provided advice and comments on the submitted SENS-IS Pre-validation Study Plan. In addition, since the test developer applied for a patent that broadly covers the test method, EURL ECVAM suggested exploring the impact of this patent on the development of similar tests, since this would have consequences on the possible future development of an OECD Test Guideline on the SENS-IS assay.

3.7.4. LuSens

The LuSens is an *in vitro* test method designed to discriminate between skin sensitising and non-sensitising chemicals. The test method is based on the same concepts as the OECD adopted KeratinoSens™ (TG 442D) by quantifying luciferase gene induction as a measure of the activation of the Keap1-Nrf2-antioxidant/electrophile response element (ARE)-dependant pathway in a genetically modified keratinocyte cell-line (Bauch *et al*., 2012). The Lu-Sens underwent an external catch-up validation study based on the performance standards for *in vitro* skin sensitisation ARE-NrF2 luciferase test methods (OECD, 2015) and the results were submitted to EURL ECVAM during the first quarter of 2015. In the light of the results of the catch-up study, EURL ECVAM considers the method would qualify to progress into the peer-review process once the test submitter provides the clarifications requested on some aspects of the submission.

3.8. Test Method Submissions Related to Skin Irritation

3.8.1. Background to the Skin Irritation Test Submissions

All formal test submissions received in the period covered by this report concern RhE-based test methods. The submissions seek formal evaluation and validation by EURL ECVAM in reference to the Performance Standards (PS) for *in vitro* skin irritation testing (EURL ECVAM 2009; OECD TG 439, 2010). This includes requests for the evaluation of similarity of test methods prior to the execution of external PS-based validation studies as well as finished studies conducted in reference to the PS.

3.8.2. OS-REp: Open Source Reconstructed Epidermis model

In May 2014, EURL ECVAM received a full submission on the OS-REp test method validation study that had been conducted externally by the test submitter in reference to the ECVAM/OECD Performance Standards for skin irritation testing (OECD TG 439).

3.8.2.1. Background to the OS-Rep

The background of this test method has been described in detail in the previous EURL ECVAM status report. Briefly, the OS-REp assay is a 3-dimensional Reconstructed human Epidermis (RhE) model consisting of normal primary human epidermal keratinocytes that, at air-liquid interface, undergo keratinization (Pruniéras *et al.*, 1983). From this point of view, it is a typical RhE "me-too" method. Importantly, the OS-REp model is not exclusively produced and distributed by a commercial entity but follows the "open source" principle: due to the fact that all the information on constructing the skin equivalents is available, tissues can be produced by any interested end user/laboratory. However, it is understood that a company intends to commercially market the OS-REp tissue kits using their automated tissue production line ("skin factory"; see previous EURL ECVAM status report, 2014).

The protocol for tissue construction is based on the RhE model developed by Poumay and colleagues (Poumay *et al.*, 2004) who demonstrated that a RhE tissue model can be constructed using keratinocytes from adult skin collected as a result of plastic surgery (abdominoplasty) from consenting patients and, importantly, by following publicly available procedures and commercially available reagents (media, growth factors, etc.) without the need of procedures/reagents protected by IPRs. Thus, this "open source" protocol enables any researcher to reconstruct, in his/her laboratory, RhE models for investigations of epidermal biology, dermatology and toxicology at a low cost while being in full control of the production process and avoiding any problems due to commercial availability or shipment. Poumay *et al*. demonstrated that the RhE model can also be used for predictive toxicity. In particular, the authors were able to distinguish a pure skin irritant from an irritant with skin sensitising properties on the basis of the pattern of inflammatory mediators released by the tissue (Interleukin 1alpha vs Interleukin 8; measured 20h post-incubation). This indicates that this test system appropriately models key aspects of the biology of human epidermis and epidermal physiology in response to xenobiotic insult. The idea of a RhE model that comes without commercial restrictions, originally put forward by Poumay, has been taken up by the test submitter and some changes to the original tissue production protocol have been introduced. The changes were intended to improve the quality of the RhE model. The resulting RhE model was termed "Open Source Reconstructed Epidermis" = OS-Rep, alluding to the open source concept of availability of information on processes and source materials (as in the software world). Changes with respect to the Poumay model include:

1) the use of ascorbic acid 2-phosphate sesquimagnesium salt instead of ascorbic acid (vitamin C) due to its longer shelf-life time in warm cell culture medium. Vitamin C significantly improves the barrier characteristics of the reconstructed tissue (Ponec *et al.*, 1997; Pasonen-Seppanen *et al.*, 2001).

2) the exclusive use of juvenile foreskin (=male skin) instead of tissue from plastic surgery of the abdomen, presumably from male and female donors. Thus, with regards to the gender source of skin tissues for keratinocyte isolation, the OS-REp RhE is based on male

keratinocytes only. Based on the keratinocyte source and near identical SOPs, this method and others (e.g. SkinEthic, epiCS) can be termed "EpiDerm-like", in contrast to EpiSkin which uses only mammary skin tissue (=female skin).

3.8.2.2. PS-based validation study

Previously, the test submitter had organised a validation study (submitted to EURL ECVAM in 2012) that had shown problems with respect to within-laboratory reproducibility (WLR), between-laboratory reproducibility (BLR) and the specificity. Following discussions and suggestions for improving the performance of the method, the submitter had organised a second study, submitted to EURL ECVAM in 2014. This study had been designed in view of demonstrating the equivalence of the OS-REp model to the validated reference methods underlying the Performance Standards associated OECD TG 439 and, at the same time, assessing the feasibility of the open source principle: tissue production was conducted under "open source" conditions at the three participating laboratories. The kits produced in the laboratories were then used for assessing the performance of the method with respect to the 20 reference chemicals in terms of WLR, BLR and sensitivity and specificity.

EURL ECVAM evaluated the dossier and study in view of assessing:

1) The sufficiency of biological and procedural similarity of the OS-REp test method in relation to the PS specifications based on the validated reference methods. Sufficient similarity is a prerequisite for a test method to qualify for the lean validation procedure via Performance Standards (using a small set of pre-defined test chemicals). The evaluation confirmed that the OS-REp method is in full compliance with the PS specifications.

2) Adequacy of the two SOPs relating (i) to the construction of the skin model in the user laboratory and (ii) to the execution of the Skin Irritation Test (SIT) for assessing the skin irritation potential of chemicals. Both SOPs were found appropriate and sufficiently detailed. The overall success of the study demonstrates that the tissue construction SOP appears appropriate. However, the study conditions can be considered favourable with regard to successful tissue constructions as extensive training had been organised for all participating laboratories. It remains to be seen how successful transfer of the tissue construction can be ensured under realistic conditions of use of the method (i.e. open source).

3) Compliance check of the validation study data with the target values of the PS relating to WLR (at least 90% concordant predictions of three valid runs per chemical), BLR (at least 80% concordant predictions among laboratories based on the mean of viability of the three runs in each lab) and PC based on assessing the individual laboratory predictions (optimally n=60 = 3 labs x 20 RCs) obtained by the mean viability of the runs generated. Specificity should be at least 70%, sensitivity at least 80%). This step involves cross checking the reported values by re-calculating the figures based on the rules set-out in the PS.

Although the study was found to be of very high quality and complied with the PS criteria with respect to BLR and predictive capacity (sensitivity, specificity), the performance for WLR were, in two laboratories (85% in one lab and 80% in a second lab) below the criteria as specified in the PS (≥90%). It would need to be demonstrated that the WLR levels that have been repeatedly shown to be attainable by other methods can also be reached using the OS-REp method which relies on tissue constructions at individual sites. This is necessary for eventual inclusion in an internationally recognised test guideline (e.g. OECD 439). EURL ECVAM has provided extensive advice to the submitter with respect to potential factors that could have caused problems with regard to WLR.

3.8.3. Sterlab Reconstructed human Epidermis

In October 2014, EURL ECVAM received a pre-submission of the Sterlab Skin Irritation Test (SIT). After evaluation by EURL ECVAM, the submitter was invited to submit a full submission which arrived at EURL ECVAM in June 2015. The full submission included results of a PS-based validation study that had been conducted in three different laboratories with the twenty reference chemicals of the PS. No other additional chemicals were tested. The Sterlab RhE SIT was submitted in view of its possible inclusion in OECD TG 439. The dossier is currently under evaluation at EURL ECVAM where the assessment of similarity and compliance with PS requirements is carried out.

3.8.4. Ashland Reconstructed human Epidermis

In May 2015, EURL ECVAM received a pre-submission of the Ashland Skin Irritation Test (SIT). This test is based on Reconstructed human Epidermis (RhE) tissues. EURL ECVAM currently assesses the pre-submission

3.9. Test Method Submissions Related to Eye Irritation

3.9.1. Ocular Irritection®

The *in vitro* macromolecular test method Ocular Irritection® represents a refinement of the former Eytex® method (Kelly, 1989; Gordon, 1992) following recommendations made by Balls *et al.*, (1995). It predicts the ocular hazard effects of chemicals based on the premise that corneal opacity may result from the disruptive effects ocular irritants may have on the highly organized structure of corneal proteins and carbohydrates. This assay mimics the biochemical phenomena of corneal protein denaturation and disruption caused by irritant chemicals acting on the cornea. One of the components of the test method is a macromolecular reagent composed of a mixture of proteins, glycoproteins, carbohydrates, lipids and low molecular weight components. The constituents of the macromolecular reagent combine with each other to form a complex macromolecular matrix that mimics the highly ordered structure of the transparent cornea. It is believed that irritant chemicals produce a turbidity of the macromolecular reagent by promoting protein denaturation, protein unfold and change in conformation, which then results in the disruption and disaggregation of the highly organised macromolecular reagent matrix. This mechanism is believed to mimic the disruptive effects ocular irritants can have on the highly organised

structure of corneal proteins and carbohydrates, which result in corneal cloudiness/opacity in the *in vivo* Draize eye test.

The Ocular Irritection® underwent an external prospective and retrospective validation study to assess its scientific validity to identify chemicals not requiring classification for serious eye damage/eye irritation (No Category) and chemicals inducing serious eye damage (Category 1), according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (UN, 2013) and the European Union Regulation on Classification, Labelling and Packaging of chemicals (EU CLP) (EC, 2008), in the framework of a Bottom-Up/Top-Down test strategy (Scott *et al.*, 2010). In December 2013, after completion of the validation study, EURL ECVAM received a full submission on the Ocular Irritection® test method. After careful evaluation, EURL ECVAM concluded that the test method appears to have promise to identify chemicals not requiring UN GHS/EU CLP classification for serious eye damage/eye irritation (e.g. as an initial step of a bottom-up testing strategy) and chemicals inducing serious eye damage (e.g. as an initial step of a top-down testing strategy) since its performance seems to be comparable to that of other validated test methods and also because it is a highly cost-effective assay as compared to other methods. However, EURL ECVAM's evaluation uncovered a number of shortcomings in the method definition and in the information provided concerning its performance and, in August 2014, the test submitter was requested to address and clarify these issues in a revised submission. Upon request of the test submitter, a meeting between EURL ECVAM and the Validation Management Group of the Ocular Irritection® validation study was organised in October 2014 to discuss the various points raised by EURL ECVAM in its assessment report. The submission was then updated with additional (non-testing) information and biostatistical analyses as requested by EURL ECVAM and the full revised submission was provided to EURL ECVAM in April 2015. EURL ECVAM currently evaluates this revised submission. If considered complete and ready to enter peer-review, it will be submitted to ESAC. Peer-review of the Ocular Irritection® test method by ESAC is expected to occur in Q4 2015 – Q1 2016.

3.9.2. Cornea Cell Reprogramming (CORNiPSC)

The Cornea Cell Reprogramming (CORNiPSC) test method was submitted to EURL ECVAM for the eye irritation endpoint. For this purpose, the test developer devised a differentiation protocol of induced pluripotent human stem cells to yield cells differentiated into human corneal epithelial cell lines (Shalom-Feuerstein *et al.*, 2012). For the measuring of eye irritation effects, the test method uses cell viability as an endpoint measurement. The test method would represent another cell-based test method for eye irritation testing, if proven to be scientifically valid, it could also have an impact on 3Rs. Since the test method is based on cell culture, it is expected that only water-soluble chemicals could be tested. This test method is in a pre-submission stage. Further refinement of the testing protocol in the light of the addressed key endpoint, will show whether this test method could be used as an initial step of an integrated testing strategy, following either a "bottom-up" or a "top-down" approach.

3.10. Test Method Submissions Related to Human Hepatic Metabolic Clearance

As part of wider efforts to develop harmonised standards for *in vitro* ADME methods EURL ECVAM has launched a public web survey asking for submission of *in vitro* human hepatic metabolic clearance methods (HHMC) in March 2014. To reach the scientific community, the survey was advertised in international scientific journals and it was disseminated through EURL ECVAM's networks, such as the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL), DataBase on ALternative Methods (DB-ALM), Preliminary Assessment of Regulatory Relevance (PARERE), ECVAM Stakeholder Forum (ESTAF), International Cooperation on Alternative Test Methods (ICATM) and through the European Society for *In vitro* Toxicology (ESTIV).

Nine test facilities submitted 15 HHMC methods that aim to measure *in vitro* the rate at which a test chemical is metabolised by a human liver based test system. HHMC methods are currently used for the prediction of *in vivo* hepatic metabolic clearance of pharmaceuticals, and their relevance to chemical testing can be initially demonstrated by employing a test system that shows strong resemblance of *in vivo* human liver. Three protocols were submitted using human liver microsomes as a test system. Liver microsomes consist of vesicles of hepatocyte endoplasmatic reticulum and thus contain almost only CYP and Uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes. One protocol was submitted using S9 fractions. This test system contains both microsomal and cytosolic fractions. However, it has low enzymatic activity. Ten protocols were submitted using human cryopreserved hepatocytes as a test system. Human hepatocytes possess the full spectrum of metabolising enzymes, co-factors and cell membrane receptors. In seven of these ten protocols human hepatocytes were applied in suspension, in two protocols hepatocytes were applied in adherent monolayer form and in one protocol, up-regulated hepatocytes were applied in adherent monolayer form. Finally, one experimental protocol using human liver slices as a test system was submitted.

Following the evaluation of the submitted methods EURL ECVAM decided to use the three most highly ranked protocols. These protocols employ cryopreserved human hepatocytes in suspension and they will be used to develop a EURL ECVAM *in vitro* hepatic metabolic clearance standard operating procedure of a representative HHMC method. This method will aim to predict the *in vivo* intrinsic hepatic metabolic clearance of fast to medium cleared chemicals.

4. Validation of Alternative Methods

This section refers to validation studies which are either carried out by EURL ECVAM or for which EURL ECVAM has been consulted. It is not an exhaustive list of all possible validation studies carried out around the world. Nonetheless, EURL ECVAM is aware of external (to EURL ECVAM) validation studies that are carried out by companies/industry on methods which are meant to be submitted to EURL ECVAM for peer review. These studies are

mentioned under chapter 3 (Test Submissions) and chapter 4.6 (EURL ECVAM Scientific Advisory Committee Activities). The validation studies undertaken with EURL ECVAM's international partners in the framework of the International Cooperation on Alternative Methods (ICATM) are described under chapter 7 (International Cooperation on Alternative Test Methods).

4.1. AR Calux

The AR CALUX assay makes use of osteosarcoma cells, stably transfected with a human androgen receptor, which express luminescence when presented with chemicals that have (anti)androgenic potential. The method was submitted by the Dutch company BioDetectionSystems for an EURL ECVAM coordinated validation study. After a positive evaluation, EURL ECVAM carried out an experimental assessment of the method, followed by a GLP study in order to refine the assay and establish transfer criteria. Three members of the 2014 established European Union Network for the Validation of Alternative Methods (EU-NETVAL) were chosen to participate in the validation study. A Validation Management Group to provide oversight on the study has been established too. EURL ECVAM provided training on the method for the 3 participating test facilities at the JRC Ispra premises early 2015, and the transfer phase of the method is currently being carried out. This assay, and other similar ones currently under validation by other validation bodies or by industry, has potential to be annexed to a future Performance Based Test Guideline (PBTG) for Androgen Receptor Transactivation Assays (ARTAs). In fact, a project to develop a PBTG and related Performance Standards on ARTAs (under the lead of the European Commission represented by JRC-EURL ECVAM) was inserted in the OECD work plan in 2013. At the present time, three ARTAs will be considered for the development of this PBTG: the AR-CALUX, the AR STTA using the AR-EcoScreen cell line (led by Japan) and the ARTA using the 22Rv1/MMTV cell line (the validation study led by Korea is currently ongoing). EURL ECVAM participates in the latter study as a member of the VMG, providing support with the chemicals selection and study design.

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). For the micronucleus test in 3D epidermis model, a slightly modified version of the former protocol is under evaluation; while for the comet assay in full-thickness skin models the between-laboratory reproducibility and predictive capacity is assessed with further chemicals.

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. 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; alternative method to the histamine sensitization test (HIST) for acellular pertussis vaccines; a multi-dose serological assay for rabies vaccine for veterinary use; *in vitro* methods for the testing of *Clostridium septicum* vaccines) or are planned for 2015 and beyond (e.g. ELISAs for tetanus/diphtheria vaccines, the BINACLE assay for *in vitro* detection of toxicity in tetanus vaccines).

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

Two projects are of particular interest since they are close to finalisation and the methods assessed are considered ready for product-specific validation at manufacturer level. Results of the above mentioned study on alternative methods to the HIST have been reviewed and discussed by regulators and manufacturers at a recent international workshop "In search of acceptable alternatives to the murine histamine sensitization test (HIST): What is possible and practical?"[27]. Participants concluded that the "indirect CHO-cell based assay" is a suitable alternative for replacement of HIST. As described by Isbrucker *et al.,* (2014), this assay had been identified as most promising in a collaborative study involving a wider range of cell-based assays and biochemical methods.

The second project, *in vitro* methods for the testing of *Clostridium septicum* vaccines has been launched within an EPAA project and more details are given under 5.12.5 (EPAA projects).

4.5. EU NETVAL

EURL ECVAM has established a network of highly qualified laboratories (EU-NETVAL) to (1) respond to some of the provisions of Directive 2010/63/EU, (2) generate *in vitro* method information that is reliable, relevant and based on current best quality and scientific

[27] https://www.nc3rs.org.uk/events/search-acceptable-alternatives-murine-histamine-sensitization-test-what-possible-and

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 (Coecke *et al*., 2014) for regulatory use in human safety assessment. The first pilot 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). Future tasks for EU-NETVAL will be the generation of high quality data based on current best scientific practices for *in vitro* methods targeting one of EURL ECVAM's priority areas, e.g. toxicokinetics (see 8.3). To actively trigger test submissions for these priority areas, EURL ECVAM identifies *in vitro* methods using test submission e-survey tools as a parallel process to the traditional test submission procedure. The whole process is managed by EURL ECVAM and its collaborating ad-hoc expert teams who analyse the information retrieved from these surveys and ultimately define internationally accepted harmonised *in vitro* method standards. EURL ECVAM is in the planning phase of involving EU-NETVAL in harmonising and standardising human hepatic metabolic clearance methods to increase reliability of this class of *in vitro* assays to use in regulatory decisions (see 3.10).

4.6. EURL ECVAM Scientific Advisory Committee (ESAC) Activities

4.6.1. ESAC Peer Review on the epiCS® Test Method for Skin Irritation Testing

From April 2014 to October 2014, ESAC reviewed the Performance Standards-based validation study on the epiCS® test method for skin irritation testing. The epiCS® test method is based on Reconstructed human Epidermis (RhE) and sufficiently similar with regard to the essential test method components to validated reference methods (e.g. Episkin and EpiDerm, OECD TG 439, see ECVAM status report 2014). The study was conducted by the test method supplier (CellSystems, Germany) and submitted to EURL ECVAM for evaluation and ESAC peer review. In agreement with the Performance Standards, the study addressed within and between laboratory reproducibility and predictive capacity on the basis of the 20 Reference Chemicals. Like most methods covered by TG 439, the epiCS® test method (initially trademarked and evaluated as EST1000) was developed to discriminate between irritant (Category 2) and non-classified chemicals in order to allow classification of chemicals according to the United Nations Globally Harmonized System (GHS) either as "Category 2" or "no Category" respectively.

The study was conducted in two phases. In 2011, data from a completed ring trial had been submitted to EURL ECVAM. While the study data met the predictive capacity target values (sensitivity = 93%, specificity = 73%), within laboratory reproducibility was not acceptable in the three laboratories involved (in particular in the naïve laboratory) and between laboratory reproducibility was slightly below the target. The submitter identified poor proficiency (no training phase had been conducted) and lack of stringency of SOP execution (in particular washing/rinsing) as likely main reasons for poor reproducibility.

Additionally, issues with shipment were identified in the case of the overseas laboratory. After improved training of the participating laboratories, all 20 chemicals were tested again in the naïve laboratory to assess whether proficiency indeed had caused within laboratory reproducibility issues. Moreover, the other two laboratories also re-tested those chemicals that had shown non-concordant predictions in view of generating supplementary information on the plausibility of the problems identified and effectiveness of the measures taken to address the problems (these were 6 and 7 chemicals, respectively). This data set showed that the measures significantly improved WLR in all three laboratories. In the case of the naïve laboratory, where all 20 reference chemicals had been tested *de novo*, WLR now met the acceptance value. The study was submitted in 2013 to EURL ECVAM and forwarded by EURL ECVAM to ESAC for scientific peer review in 2014.

ESAC reviewed the study design and data sets submitted in 2011 and in 2013 and, in an interim report, concluded that the study showed acceptable values for BLR, specificity, sensitivity and overall accuracy. However, only the naïve laboratory satisfied the performance values relating WLR when re-testing all 20 reference chemicals in 2013. The two other laboratories only met performance values when grouping data from the 2011 testing with data from the re-testing in 2013. Although these were generated under slightly different conditions with regard to exactitude of SOP execution, ESAC was of the opinion that this was not an *a priori* reason to reject such grouping which can indeed be a valid approach in agreement with the modular approach, in particular with respect to use of retrospective data. Although ESAC was of the opinion that all data together tended to support that performance values for WLR can be attained, ESAC was concerned that grouping the data from *de facto* two validation trials may introduce bias towards more positive results, since the remaining 14 and 13 chemicals had not been re-tested and hence had not been subjected to the possibility of generating results of lesser quality (e.g. more non-concordant or invalid runs/run sequences). ESAC recommended that for a final robust characterisation of WLR supporting regulatory use of the test method, also the remaining 13 and, respectively, 14 reference chemicals would need to be re-tested in the two participating laboratories. The submitter is currently (summer 2015) generating these missing data and the dossier is expected to go into final ESAC peer review in autumn 2015.

4.6.2. ESAC Peer Review on the hCLAT Test Method for Skin Sensitisation

From September 2013 to March 2014 ESAC reviewed the EURL ECVAM-led validation study which had assessed mainly the transferability and reproducibility (within and between laboratories) of the h-CLAT test method (primary objective of the study) in view of its possible future use as part of a non-animal testing strategy for skin sensitization. The study had also been designed to provide preliminary information on a) the predictive capacity of the test method and b) its potential use for contributing to sub-categorisation of sensitising chemicals. Overall, ESAC agreed with the conclusions drawn by the Validation Management Group that oversaw the study. The ESAC however disagreed with the VMG conclusion concerning the interpretation of the results on Within Laboratory Reproducibility (WLR) which was found to be on average 80% WLR over the four participating laboratories

and thus not meeting the expectation of the VMG (85%). ESAC expressed concern that there may be inherent characteristics or critical aspects of the h-CLAT test method, which could be sources of variability that may impact on the usefulness of the data even when generated in the context of an integrated approach. Furthermore, while agreeing that the study generated promising results, ESAC concluded that predictive capacity, applicability domain and limitations of the test method were not yet fully defined and would require further characterisation through empirical testing and/or the evaluation of existing information.

4.6.3. ESAC Peer Review on the Human-based CYP induction assays

From April 2014 to October 2014 ESAC reviewed the EURL-ECVAM coordinated validation study on cytochrome P450 induction (CYP1A2, CYP2B6, CYP2C9, and CYP3A4) in human cryopreserved primary hepatocytes and the human cryopreserved HepaRG cell line. The main study objective was to assess the transferability, reproducibility (within and between laboratories) and predictive capacity of the two *in vitro* test systems employed. Predictive capacity was assessed using exclusively human reference data. Availability of robust human *in vivo* CYP induction data was one of the criteria in chemical selection and this limited the test chemicals to pharmaceuticals. Chemicals were tested over a wide range of concentrations covering clinical human dose-response. Chemicals with animal data only were avoided because they are not comparable to the human situation due to the well described metabolic interspecies differences.

The ESAC concluded that the study had appropriately addressed test definition, within laboratory reproducibility, transferability, and between laboratory reproducibility. However, ESAC was of the opinion that the assessment of predictive capacity had been only partially fulfilled and concluded that the data as generated during the study were not fully sufficient to conclude on the readiness of the test for regulatory use supporting chemical safety assessments. In particular, ESAC recommended that additional CYP induction studies be conducted with rodent hepatocytes to investigate both applicability domain and predictive power for substances that have "drug-like" properties (i.e. persistence/bioaccumulation, highly lipophilic compounds, rapid metabolism, and poor water solubility). Notwithstanding this recommendation, ESAC agreed that CYP induction assays based on human cells may have a potential role in chemical safety assessments, e.g. as markers of possible receptor activation or as an adjunct test to interpret other toxicological information. According to ESAC, the human cell-based assay has also potential use for evaluating the human relevance of animal test results, whether *in vivo* or *in vitro*. ESAC further concluded that the study data were in good agreement with other existing information regarding hepatocyte assays and provided a proof of principle: that EURL ECVAM study had demonstrated that reliable hepatocyte assays for other important purposes, including identification of metabolites and quantification of metabolic clearance, are feasible. ESAC encouraged EURL ECVAM to continue conducting studies with human hepatic models to develop methods for characterization of other kinetic data, including

clearance, metabolic profiling, and inhibition. In this context, ESAC emphasized the importance of developing *in vitro* to *in vivo* extrapolation methods.

4.6.4. ESAC Peer Review on the EpiOcular™ Eye Irritation Test (EIT)

From March to October 2014, ESAC reviewed the EURL ECVAM eye irritation validation study (EIVS). The EIVS originally addressed two test methods, the SkinEthic™ HCE assay reconstructed cornea based on a human corneal cell line and the EpiOcular™ EIT assay using reconstructed tissue based on keratinocytes from male foreskin. During the study it became apparent that the SkinEthic™ HCE would require optimisation and further validation, which could not be completed within the time-frame of the EIVS. The ESAC review hence focused primarily on the EpiOcular™ EIT. ESAC concluded that the study addressing the potential usefulness of the EpiOcular™ EIT for use within a test strategy to assess eye irritation of chemicals was well planned and executed. Reproducibility, transferability and predictive capacity had been assessed in three laboratories testing 105 chemicals plus an additional 8 solids to assess an optimised version for testing of solids. The chemicals represented a wide range of irritancy scores, chemical categories and use classes. While ESAC agreed with the bulk of conclusions of the study results as presented by the VMG, ESAC disagreed with the approach chosen to calculate the predictive capacity based on the individual test runs generated in all participating laboratories. According to ESAC, this approach over-estimated the sample size, produced overly narrow confidence limits, and resulted in a seemingly high degree of certainty with respect to the point estimates of sensitivity and specificity. ESAC recommended that, in case single runs are used for analysing predictive capacity, other statistical methods such as bootstrapping should be used. ESAC concluded that the study had satisfied the requirements regarding transferability, reproducibility and predictive capacity, supporting the use of the EpiOcular™ EIT within a test strategy to determine the eye irritation potential of chemicals, specifically to detect non-irritants as part of a Top-Down or Bottom-Up approach. Despite this positive appraisal, ESAC pointed out that confidence in the test method would be further increased if supplementary studies confirm that the test method correctly classifies a small but representative sample of labelled products or mixtures as defined by REACH and from various sectorial use classes.

4.7. EURL ECVAM Recommendations

4.7.1. EURL ECVAM Recommendation on the Zebrafish Embryo Toxicity Test (ZFET) for Acute Aquatic Toxicity Testing

Acute fish toxicity testing is an important component of the environmental hazard assessment of chemicals. For many years, (zebra-)fish embryo-based methods have been proposed as alternatives to the acute fish toxicity test carried out with juvenile or adult fish (e.g. Schulte and Nagel 1994; Nagel 2002; Braunbeck *et al.*, 2005; Scholz *et al.*, 2008; Lammer *et al.*, 2009).

The Zebrafish Embryo Acute Toxicity Test Method (ZFET) is based on the use of newly fertilised eggs from zebrafish (*Danio rerio*). It is a short-term exposure test (96 h) and

determines the concentration that is lethal to 50% of the zebrafish embryos (LC50) as an indication of acute fish toxicity. Observation of one of the following apical endpoints indicates the death of the embryo: coagulation of the embryo, lack of somite formation, non-detachment of the tail and lack of heartbeat.

On behalf of the OECD, EURL ECVAM coordinated during 2008-2012 the validation of the ZFET to evaluate its reproducibility in support of the development of an OECD Test Guideline (OECD 2011, 2012; Busquet *et al.*, 2014). In parallel to this study, Belanger and colleagues continued to collect acute fish embryo toxicity and acute fish toxicity data to assess the predictive capacity and applicability of the ZFET and submitted their report to EURL ECVAM in July 2012 (Belanger *et al.*, 2012; 2013). Following independent scientific peer review by ESAC of both studies and having considered input from regulators, stakeholders, international partners and the general public, EURL ECVAM concluded (EURL ECVAM, 2014) that the ZFET - being available as OECD TG 236 since 2013 (OECD, 2013b) - is transferable and reproducible within and between laboratories and can provide information on acute fish toxicity comparable to that derived from standard tests (e.g. OECD TG 203; OECD 1992). Therefore, it should be used for generating information on acute fish toxicity, where appropriate taking into consideration the various regulatory frameworks and regions as well as its potential limitations.

The use of the ZFET will result in an overall reduction in the numbers of juvenile and adult fish required for aquatic toxicity testing. Notably, since Directive 2010/63/EU (EU 2010) on the protection of animals used for scientific purposes covers larval forms of non-human vertebrate animals once they are independently feeding, the ZFET as used in OECD TG 236 is outside the directive's scope: zebrafish start to feed independently not before 5 days post-fertilisation and the method uses zebrafish embryos only up to 4 days (= 96 h) post-fertilisation.

Prospective users of the method should consult EURL ECVAM's DataBase for ALternative Methods (DB-ALM) to access the detailed ZFET protocol (see DB-ALM Protocol no. 140 at the address: http://ecvam-dbalm.jrc.ec.europa.eu.

The EURL ECVAM Recommendation on the Zebrafish Embryo Acute Toxicity Test for Acute Aquatic Toxicity Testing was published in July 2014 (EURL ECVAM, 2014).

4.7.2. EURL ECVAM Recommendation on the human Cell Line Activation Test (h-CLAT) for Skin Sensitisation Testing

The human Cell Line Activation Test (h-CLAT) for skin sensitisation testing was developed by Kao Corporation and Shiseido (Japan). With a view to facilitating its use as a component of integrated approaches to assessing the skin sensitisation potential of chemicals, EURL ECVAM coordinated a validation study to assess the reliability of the h-CLAT method and to gain some preliminary insight into its predictive capacity. On completion of the study, EURL ECVAM requested ESAC to conduct a scientific peer review of the validation study report and the resulting ESAC opinion was delivered in May 2014 (see 4.6.2). Having considered

the ESAC opinion and inputs from regulators, stakeholders, international partners and the general public and taking into consideration all existing published information on the method, in February 2015, EURL ECVAM published its recommendation on the h-CLAT indicating that the test method is transferable to laboratories sufficiently experienced in cell culture techniques and flow cytometry analysis. Considering the within and between-laboratories reproducibility and the preliminary predictive capacity of the method as assessed with the chemicals tested in the validation study and in published studies, EURL ECVAM concluded that the test method should prove valuable as part of Integrated Approaches to Testing and Assessment (IATA) together with complementary information (e.g. *in chemico* or other *in vitro* data, QSAR or read-across predictions). The ESAC peer review of the h-CLAT study included valuable expert discussion of various statistical approaches to assess within and between laboratory reproducibility. As a follow-up, EURL ECVAM proposed to re-analyse the data from the validation study with a view to exploring the merits of various statistical methods for describing the reproducibility of a test method that produces a classification-based prediction. EURL ECVAM undertook this statistical analysis and produced a report that was submitted to the OECD in support of the development of a Test Guideline on the h-CLAT.

EURL ECVAM recommendations on the EpiOcular™ Eye Irritation Test (see 4.6.4) and on the human-based CYP induction assays (see 4.6.3) are currently being prepared.

5. Promoting the Regulatory Acceptance 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 2014, the meetings took place on 5th and 6th June.

5.1.1. PARERE Meeting

At the PARERE meeting on 5th June 2014, the participants gave an overview on 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.

In relation to the tasks that PARERE should carry out, the following was agreed:

1. Provide upstream input on potential regulatory relevance and suitability of proposed alternative approaches and identify approaches that deserve attention;
2. Highlight 3Rs priority areas within the regulatory domain and provide feedback on draft EURL ECVAM strategy documents;
3. Comment on draft EURL ECVAM Recommendations;

4. Identify regulatory experts that could participate in specific EURL ECVAM project groups (e.g. supporting validation studies, peer-review working groups);
5. Support and facilitate the work of the EU Network of laboratories for the validation of alternative methods (EU-NETVAL) within Member States; and
6. Contribute to the promotion, dissemination and communication of alternative approaches within Member States.

In the framework of task 1 above, EURL ECVAM consulted PARERE on the regulatory relevance of a test method for the prediction of the *in vivo* genotoxic potential of chemicals (GreenScreen™HC), which had been initially submitted in 2009 and for which an updated full submission was received in 2014 (see 3.2.1). In addition, the PARERE consultation process on a test method for teratogenicity testing (DevTox qp) was launched.

5.1.2. Joint PARERE-ESTAF meeting

Following the PARERE meeting (see above), a joint meeting of PARERE and ESTAF members was held on 5th and 6th June 2014. EURL ECVAM gave updates on its activities in relation to alternative approaches; explained modifications of its validation workflow; and informed PARERE on the establishment of the European Union Network for the Validation of Alternative Methods (EU-NETVAL). EURL ECVAM presented strategies in the following three areas:

- Toxicokinetics;
- Acute systemic toxicity; and
- Fish toxicity and bioaccumulation.

PARERE and ESTAF members commented on these draft strategies by written procedure after the meeting and the strategies were published by ECVAM[28].

Finally, participants provided their views on a number of topics including the efficiency of the review process of ESAC, the usefulness of EURL ECVAM Recommendations outside the context of OECD Test Guidelines and the possibilities of a test submitter to comment on a validation study report and the draft EURL ECVAM Recommendation in relation to their test method. In addition, the participants were asked to provide their views about considerations to implement a fully transparent model of test submissions, i.e. making public all details of a test method submitted to EURL ECVAM.

5.2. OECD Test Guideline Programme (TGP)

At the 27th meeting of the Working Group of National Coordinators of the OECD TGP held at the OECD headquarters in Paris on 14th to 17th April 2015, six new Test Guidelines were approved of which four were based on *in vitro* methods, i.e. the Short-Time Exposure test (eye hazard potential), a test method based on reconstructed Human Corneal Epidermis (eye hazard potential) and the Estrogen-Receptor Binding Assay (endocrine disruption). In

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

the context of a broader revision and update of genotoxicity OECD Test Guidelines, the mouse lymphoma assay and TK6 test (using TK gene locus), originally contained in TG 476, were moved to the new TG 490 based on revision of acceptance criteria and data interpretation. Now, two distinct TGs for *in vitro* mammalian cell gene mutation tests are available: TG 476 – using *hprt* and *xprt* genes; TG 490 – using *tk* gene.

In addition, ten Test Guidelines were updated (TG 404, TG 430, TG 431, TG 435, TG 439 on *in vitro* and *in vivo* skin irritation and/or corrosion; TG 478 and TG 483 on *in vivo* genotoxicity, TG 455 on Estrogen-receptor transactivation (endocrine disruption), TG 421 and TG 422 on screening for reproductive toxicity (updated with endocrine-related endpoints).

A series of supporting documents were approved as well. More information on the OECD TG programme can be found on the OECD website of the Test Guideline Programme[29].

The following chapters exclusively focus on TGs for which the EC (through JRC-EURL ECVAM) had the lead or co-lead or carried out validation studies. Beside those, EURL ECVAM participated in, or co-chaired, numerous OECD expert groups and commented on several draft TGs and GDs led by other OECD Member Countries.

5.2.1. OECD Test Guidelines on Skin Sensitisation

5.2.1.1. Adopted Test Guidelines

In March 2015, TG 442C on the Direct Peptide Reactivity Assay (DPRA) and TG 442D on the ARE-Nrf2 Luciferase Test Method, KeratinoSens™ were adopted by the OECD. These represent the first two test guidelines describing non-animal test methods for skin sensitisation. The DPRA assay is an *in chemico* assay that addresses protein reactivity, the molecular initiating event (Key event 1) of the skin sensitisation adverse outcome pathway (AOP), by quantifying the reactivity of test chemicals towards synthetic peptides containing either lysine or cysteine. The KeratinoSens™ test method focuses on the second key event of the AOP, the inflammatory response and gene expression in keratinocytes associated with the antioxidant/electrophile response element (ARE)-dependent pathways. Both test methods underwent EURL ECVAM validation and/or peer review. The issuing of the EURL ECVAM recommendation on the DPRA in 2013 and the one on the KeratinoSens™ in 2014 formed the basis for the development of OECD test guidelines. Both methods are proposed in the test guidelines for supporting the discrimination between skin sensitisers (i.e., UN GHS category 1) and non-sensitisers within Integrated Approaches to Testing and Assessment (IATA).

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

5.2.1.2. Draft OECD Test Guideline on h-CLAT

At OECD, EC-EURL ECVAM is co-leading together with Japan the development of a third test guideline on an *in vitro* method for skin sensitisation validated by EURL ECVAM. The method in question is the human Cell Line Activation Test (h-CLAT) which is addressing the process of dendritic cells activation, the third key event within the skin sensitisation AOP. A draft test guideline was submitted to OECD in 2014 and underwent revisions following the first commenting round. In addition EURL ECVAM submitted to the OECD a report on the reanalysis of the validation study data where the reproducibility of the method was reconsidered by looking at all possible combinations of the nine conducted runs per chemical, rather than the sequential analysis initially performed. At the 27th WNT meeting, it was agreed that the reanalysis has been useful to address some of the issues raised by the WNT comments and there was general support to move the TG towards approval once the remaining minor issues have been discussed and solved. A second commenting round took place shortly after the 27th WNT meeting and an OECD meeting of the expert group on skin sensitisation will be held in October 2015 to address the comments received with a view of moving the TG forward for written approval by the WNT.

5.2.1.3. New OECD Project Proposals for developing Test Guidelines on *in vitro* methods for skin sensitisation testing

U-SENS™

Further to the evaluation of an industry-led validation study and submission of the information to EURL ECVAM (see 3.7.2), a project proposal for the development of a Test Guideline on the U-SENS™ test method was submitted by France to the OECD and included in the OECD work program in 2015.

IL-8 Luc assay

A project proposal was made by Japan for developing a Test Guideline on the IL-8 assay (see 7.2) and included in the OECD work program in 2015. The test method was evaluated in a JaCVAM-coordinated validation study which was finalised in September 2014. The independent peer review by JaCVAM of the validation study started in February 2015 and is currently being finalised..

5.2.2. OECD Test Guideline on reconstructed Human Corneal Epidermis for the detection of chemicals not requiring classification for serious eye damage/eye irritation

Based on the outcome of the EURL ECVAM/Cosmetics Europe validation study (see EURL ECVAM status report 2014), the EC (through JRC-EURL ECVAM) submitted a new draft TG on the EpiOcular™ EIT for the identification of chemicals not requiring classification for serious eye damage/eye irritation to the OECD in July 2014. After a first commenting round, a meeting of the expert group was convened at the OECD headquarters in Paris in November 2014 to discuss and address the comments from experts and the WNT on the first version of the TG. Following this meeting, a revised draft TG and associated Performance Standards (PS) were prepared by EURL ECVAM and circulated to the WNT and the OECD expert group on eye irritation for commenting in December 2014. The revised

documents were finally submitted to the OECD for approval in February 2015 and were adopted by the OECD WNT in 2015. The TG (no. 492) and PS were published by the OECD in July 2015. TG 492 was published in Section 4 (Heath Effects) of the OECD Test Guidelines and the PS in the Series on Testing and Assessment.

5.2.3. OECD Guidance Documents on SHE and Bhas 42 CTA

Cell transformation assays provide a way to detect, in cultured cells *in vitro*, those phenotypic alterations that have been considered alterations associated with cells exhibiting neoplastic potential *in vivo.* A draft TG on the cell transformation assay (CTA) in SHE cells had been submitted for adoption at the 26th WNT meeting in 2014. As there was no consensus to approve the revised TG due to a minority of Member Countries who were against it, it was agreed to make the SHE CTA test method available in a guidance document (GD). This GD has been published as No. 214 in the Series on Testing and Assessment in May 2015. As in the case of the SHE CTA, the WNT also decided that the draft TG on the other version of the CTA, based on BHAS 42 cells, should also become a GD. The draft GD underwent several commenting rounds and the revised version will be sent to the WNT for a final review before approval and declassification by the Joint Meeting.

GDs are not covered by Mutual Acceptance of Data (MAD), but they will allow test method users to perform the test methods in a standardised manner. This will hopefully lead to the generation of new data of good quality, which will in the future be useful for the revision of the status of the assays.

In April 2014, the WNT recognised that the CTA is insufficient to completely address non-genotoxic carcinogens and that a more comprehensive battery of tests addressing non-genotoxic mechanisms was needed. This discussion raised the need for an IATA approach to properly address the issue of non-genotoxic carcinogenicity and discuss where the CTA, together with other relevant assays, could fit. A new project proposal by the UK for the development of an IATA for non-genotoxic carcinogens was approved at the 27th WNT. The project will provide a thought starter that examines what non-genotoxic carcinogens are, the current regulatory difficulties encountered with respect to them and how an IATA could be explored and ultimately developed in the second stage of the project to assist regulators in their assessments of non-genotoxic carcinogenicity.

5.2.4. Draft OECD Performance-based Test Guideline on CYP induction

Further to the EURL ECVAM-coordinated validation study on human-based CYP induction assays (reported in the EURL ECVAM status report 2014) and the subsequent ESAC peer review (see 4.6.3.), the EC (JRC-EURL ECVAM) developed and submitted a draft Performance-based test guideline (PBTG) on "human cytochrome P450 (CYP) activity n-fold induction" to the OECD in July 2014. The comments raised by the WNT in this new area of *in vitro* biotransformation assays were reviewed and addressed in an OECD expert group meeting on biotransformation assays in May 2015.

The submitted PBTG was considered to fit well within the new OECD concept for advancing test methods dealing with complex endpoints. The information generated by the human CYP activity n-fold induction *in vitro* test method may be included in integrated approaches to testing and assessment when attempting to assign a test chemical to a particular Adverse Outcome Pathway. CYP induction can be identified as a molecular initiating event (AOP-dependent; e.g. cholestasis and CYP3A4 up regulation) or may be of relevance because induction may alter key metabolic pathways by perturbing the biotransformation pathways catalysed by the induced CYP enzyme (AOP-independent).

The expert group recognised the usefulness of the submitted PBTG not only to assess potential CYP induction of test chemicals but also of chemicals in mixtures which are likely to significantly change metabolic pathways, i.e. change the biotransformation rates of individually co-occurring substances and their metabolites with potential consequences for their toxicity.

Based on the results generated during the validation project, the expert group considered the human cytochrome P450 (CYP) activity n-fold induction *in vitro* test method robust and reliable and supported the submitted PBTG. However, to better explain the context of use of this type of PBTG or of a new TG, an explanatory background document that will accompany the PBTG, will be drafted. All the experts at the meeting agreed to contribute to this document for progressing the PBTG.

In general, the approach adopted by EURL ECVAM for the development of standards for ADME *in vitro* methods aimed at human risk assessment was appreciated. The development of other PBTGs covering *in vitro* methods on human hepatic metabolic clearance, but also on protein binding and oral/dermal and inhalation barrier routes useful for the assessment of bioavailability of compounds, is encouraged.

5.2.5. Development of an OECD TG "*In vitro* Fish Hepatic Metabolism"

In April 2014, the OECD WNT approved a project (under the lead of USA and the European Commission represented by JRC - EURL ECVAM) on the development of a new OECD TG on *In vitro* Fish Hepatic Metabolism. The project aims at standardising two *in vitro* methods using rainbow trout S9 fraction (Johanning *et al*., 2012) or cryopreserved rainbow trout hepatocytes (Fay *et al*., 2014) to determine *in vitro* fish intrinsic hepatic clearance rates.

A multi-laboratory ring trial to assess the reliability, transferability, and predictive value of the two *in vitro* methods started in late 2014 and will provide results in 2015. It builds on work carried out within the framework of the ILSI HESI project "Bioaccumulation". ILSI HESI, will coordinate the ring trial and the outcome will serve as basis for developing the OECD TG.

The bioconcentration potential of a chemical is important information that is required in many pieces of chemical legislation. It is used for hazard classification and for the assessment of persistent, bioaccumulative and toxic (PBT) substances. The

Bioconcentration Factor (BCF) is either predicted or measured (typically in fish, but if necessary, also in invertebrates).

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

5.2.6. New OECD Project Proposals in Fish Toxicity

Control fish

Following up on discussions initiated by the International Council on Animal Protection in OECD Programmes (ICAPO) at the 25th WNT meeting in 2013, the European Commission (JRC - EURL ECVAM) and ICAPO submitted a project proposal to OECD addressing 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, i.e. a water control and a solvent control. Part 1 of the project aims at minimising the use of solvents and 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. 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.

5.2.7. New OECD Project Proposal for a Guidance document on an IATA for serious eye damage/eye irritation

The EC (JRC-EURL ECVAM) and the United States Environmental Protection Agency (US EPA) jointly submitted a new project proposal for the development of a Guidance Document on an IATA for serious eye damage/eye irritation (see 5.3.3.1) to the OECD in November 2014. This new project proposal was discussed and approved by the OECD WNT at its 27th meeting in 2015.

5.3. Integrated Approaches to Testing and Assessment (IATA) developments

5.3.1. General background on IATA

IATA can be defined as a framework used for hazard identification, hazard characterisation and/or safety assessment of a chemical or group of chemicals, which strategically integrate and weight 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. At the OECD level, IATA projects are run either under the OECD Test Guideline Programme or the OECD Task Force on Hazard Assessment.

5.3.2 Guidance Document on the Reporting of IATA

EURL ECVAM is leading at the OECD the development of two guidance documents (GD), one on the reporting of IATA and one on the reporting of structured approaches to data integration and individual information sources used within IATA for skin sensitisation. The overall purpose of these GDs is to promote the consistent reporting of IATA within OECD member countries. The first guidance document delivers a set of principles for describing and evaluating IATA to facilitate their consideration in regulatory decision-making. In addition, the GD provides templates for reporting: a) structured approaches to data integration and b) individual information sources used within IATA so that the same documentation format for describing and evaluating IATA and its elements is used to the extent possible.

These templates were used by an ad-hoc expert group for developing the second guidance document in which a number of structured approaches to data integration are presented. These can constitute, or be part of IATA for skin sensitisation hazard and potency prediction, based on the adverse outcome pathway as a conceptual framework. Among the case studies, the approach developed by EURL ECVAM is also presented.

This document does not intend to seek for endorsement of any specific approaches provided in the case studies, but rather to provide a perspective of how individual information sources and structured approaches to data integration used within IATA for skin sensitisation should be reported in a harmonised way and to illustrate what forms these may take, whether they are statistically derived, or qualitative in nature, and serve different purposes (i.e. hazard versus potency prediction). A harmonised approach in reporting is critical to ensure consistency in the use of IATA-derived predictions/assessments for regulatory decisions and to promote mutual acceptance of such assessments.

The two GDs are currently under review by the OECD Hazard Assessment Task Force (HATF).

5.3.3. IATA Development in the OECD Test Guideline Programme

EURL ECVAM co-leads and contributed to two IATAs which were developed in the OECD Test Guideline Programme: the IATA on Serious Eye Damage/Eye Irritation and the IATA on Skin Irritation/Corrosion.

5.3.3.1. Development of an OECD Guidance Document on an Integrated Approach to Testing and Assessment (IATA) on Serious Eye Damage/Eye Irritation

Assessments of serious eye damage and eye irritation hazards are basic information requirements in international regulations for the safety of chemicals, pesticides, and medicines including classification and labelling, packing and their transport. 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 (OECD, 2002). This strategy recommends that, prior to undertaking the described *in vivo* test, a weight-of-evidence analysis be performed on all existing relevant data and that, where sufficient data are not available, new data need to be developed through application of a sequential testing strategy, starting first with alternative methods, in order to avoid unnecessary testing in laboratory animals. Steps 5 and 6 of this sequential testing and evaluation strategy call for validated and accepted *in vitro* or *ex vivo* test methods for "eye corrosion" and "eye irritation", respectively, before the use of the *in vivo* OECD TG 404 (on acute skin irritation/corrosion) and the *in vivo* TG 405 (on acute "eye irritation/corrosion") in steps 8 and 9, respectively, with the purpose of minimising animal use. However, this strategy does not foresee the use of negative results from validated and accepted *in vitro* assays, requiring confirmatory *in vivo* testing in such cases. Since publication of the supplement in 2002, several *in vitro* methods for serious eye damage/eye irritation have been developed, validated and accepted by the OECD. Depending on country requirements and the results obtained with the OECD accepted methods (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) that could contribute to the full replacement of the *in vivo* Draize 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 update in view of amending the possible use and usefulness of individual test methods and in order to avoid contradiction between the provisions of individual OECD TGs on *in vitro* methods and the provisions of the OECD TG 405 supplement.

The EC (JRC-EURL ECVAM) and the US EPA thus proposed to develop a Guidance Document on IATA for serious eye damage/eye irritation that should provide guidance on how to use the various types of alternative methods available and 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 has three main objectives:

1. To propose an IATA considering the scientific and technical progresses in the development, validation and acceptance of alternative methods to assess serious eye damage/eye irritation, in view of replacing the "testing and evaluation strategy" provided in the supplement to OECD TG 405, which was developed in 2002.
2. To provide a clear and consistent description of each of the individual information sources that are relevant for the assessment of serious eye damage/eye irritation in the context of an IATA in terms of their key performance characteristics (reproducibility and predictive capacity) and their usefulness and limitations.
3. To provide guidance on how to best use and combine relevant information in the context of the IATA for regulatory decision making on the serious eye damage or

eye irritation hazard potential of test chemicals (including decisions on the need for further testing when the existing information is not sufficient to reach a sound decision).

A first draft of the document should be available for discussion at a meeting of the OECD Expert Group on eye irritation planned for November 2015 at the OECD headquarters. The final document is expected to be submitted for approval by the OECD WNT in April 2017.

5.3.3.2. Guidance Document on an Integrated Approach to Testing and Assessment for skin corrosion/irritation hazard identification

In July 2014, the OECD published the guidance document on an "integrated approach on testing and assessment" (IATA) for skin corrosion and irritation hazard identification (series on testing and assessment No. 203) (OECD, 2014). This represents an important milestone in the area of skin corrosion/irritation assessment. The document was developed by the OECD expert group for skin corrosion and irritation with substantial contribution from EURL ECVAM experts. Building on the Integrated Testing Strategy (ITS) for skin corrosion/irritation assessment which had been developed in 2006/2007 in the context of implementation of the REACH legislation, the OECD IATA outlines a strategic and scientifically based workflow to assess the hazard of a wide spectrum of chemicals in view of concluding on their classification and labelling (C&L) as well as choosing appropriate packing groups for transport. Like the original REACH ITS, the OECD IATA provides a step-by-step approach that is organised in three fundamental blocks: (1) gathering of existing information and conduct of weight of evidence of individual data sources; (2) weight of evidence of all collected information and, if necessary, an analogue approach; (3) new testing using first non-animal test methods and employing a strategic approach based on whether the substance is likely to be hazardous (either irritant or corrosive) or whether it is more likely to be non-hazardous. Animal testing is only foreseen as a last resort. While preserving this fundamental approach, additional data sources have been added also taking into account progress that has been made with respect to the capacity of alternative methods to provide additional C&L information. For instance, the IATA now supports subcategorisation of corrosive substances in subcategory 1A and a combination of subcategories 1B and 1C. As a novelty, it makes provision for using information from non-guideline methods, in particular where such methods can provide additional information that may be needed depending on country requirements. This includes for instance information on Category 3 ("mild" irritants) or full subcategorisation of skin corrosives (that is resolving subcategories 1B versus 1C). Thus, with these flexible instruments, the IATA allows to completely replace the traditional Draize rabbit test for skin corrosion / irritation (OECD Test Guideline 404) for a wide spectrum of chemicals/use classes.

While the IATA is a big step forward, there is still considerable room for improvement in the area of skin corrosion/irritation. We outline the most important in the following:

Importantly, while the current IATA provides solutions for classification and labelling needs of all global areas, it does so by resorting to non-guideline test methods or protocols, often relying on one single alternative method. This is far from ideal. It is therefore recommended that also other test method developers invest in improving their methods / protocols in view of providing data on corrosion subcategorisation and also on Category 3 predictions. Recently, progress with regard to improving the prediction model of some of the RhE based corrosion models have been made, allowing more accurate subcategorisation predictions (Desprez *et al.*, 2015).

Moreover, it should be noted that the skin irritation methods are all based on the very upstream readout of tissue trauma (a key event in dermal inflammation, see provisional AOP for skin irritation in Worth *et al.*, 2014, but do not exploit more mechanistic parameters such as inflammatory mediators or other stress indicators.

Finally, when improving prediction models for categorical predictions, it would be advisable to reflect on the introduction of the prediction "inconclusive" for chemicals that fall within defined margins close to the cut-off of the prediction model (further details in Griesinger *et al.*, 2015) since the categories of C&L are entirely arbitrary and put on a continuum of effect from non-irritant to irritant. A more scientific approach would be to process borderline predictions (falling within said margins) by expert judgement to decide on the final classification or non-classification.

5.4. OECD Good Laboratory Practices Programme: EURL ECVAM Good In Vitro Method Practice (GIVIMP) Project

In vitro methods, often based on the use of human cells and tissues, are submitted to international validation bodies. Well-designed, robust, reliable *in vitro* methods that can be run in a routine environment for generating data sets are becoming more and more instrumental for supporting regulatory decisions. Therefore, a project on an international 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 launched by EURL ECVAM. This project has recently been included in the OECD Test Guideline work programme. The major goal of these efforts consists of reducing the uncertainties in *in vitro* based predictions and therefore increasing the acceptance of the *in vitro* estimated safety measures by regulatory agencies. The GIVIMP guidance is meant to gather expert input from regulators of European and international authorities such as the European Food Safety Authority, the European Medicines Agency, the European Chemicals Agency, the US Food and Drug Administration as well as from the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL), from ECVAM's Stakeholder Forum, from the EU and OECD Working Group on Good Laboratory Practices, from European 3Rs Centres, from EURL ECVAM's Preliminary Assessment of Regulatory Relevance (PARERE) network, from scientists from large industries and small and medium-sized enterprises and from international scientists with expertise in kinetics, stem cells, cell biology, GLP and *in vitro* methods. The purpose of the GIVIMP guidance is taking into

account all necessary good scientific, technical and quality practices, to ensure that the overall process from *in vitro* method development to *in vitro* method implementation for regulatory use becomes more efficient and effective. An experimental project will be undertaken as a first GIVIMP-based pilot project aiming at harmonising and standardising human hepatic metabolic clearance methods to increase reliability of this class of *in vitro* assays to use in regulatory decisions.

5.5. Support to OECD Policies: Chemical Mixtures and Combined Exposure

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

Thus, one of the objectives of recent EURL ECVAM work has been to gain a clear up-to-date overview on the current regulatory requirements and available guidance, as well as to collate information on the application of different approaches in current risk assessments. To this end, more than 20 different pieces of EU legislation (food and non-food related such as REACH, plant protection products, biocides, medicines, cosmetics, food contaminants, food and feed additives etc.) were reviewed as well as guidance documents from the EU and international bodies (Kienzler *et al*., 2014). Furthermore, an expert survey was performed to gather information on current practices and expert views.

In conclusion, while many pieces of EU legislation are in place to protect humans and the environment against adverse effects of chemicals including mixtures, in many cases it remains unclear how this is to be carried out and only few explicitly consider (real life) exposure to mixtures. In cases where mixtures are considered, the assessment is frequently limited to some well-known components. Several mathematical models and approaches have been developed to assess the toxicity of mixtures, but their routine application is hampered by considerable information gaps.

To further investigate this question, and 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. Contributions to the OECD Adverse Outcome Pathway development Programme

In 2012, the OECD launched a new programme on the development of Adverse Outcome Pathways[30] (AOP). AOPs are structured, mechanistically-based descriptions of chemically-induced toxicity. They portray existing knowledge concerning the linkage between two anchor points, the Molecular Initiating Event (MIE), and an Adverse Outcome (AO), connected by a chain of Key Events (KE) and the relationships between them (KER). AOPs are typically represented sequentially, moving from one KE to another, as compensatory mechanisms and feedback loops are overcome. These KEs are a limited number of measurable and toxicologically relevant events that are essential for the progress to the AO. An AOP is not required to provide a comprehensive molecular description of every aspect of the biology, focusing instead on the critical steps in the pathway.

While there are a large number of cellular and molecular processes known to be critical for proper development and function of the different systems in the body, there are relatively few examples of a comprehensive understanding of how these processes are perturbed following a chemical exposure leading to an adverse outcome. AOPs have the potential to become a powerful tool to support alternative methods for chemical risk assessment which may be predictive of the adverse outcome *in vivo* without the need to actually demonstrate the adverse outcome.

EURL ECVAM has undertaken the following AOPs projects relevant to neurotoxicity (see 8.4), toxicity via disruption of the endocrine system and liver toxicity (see 2.1). For five of the seven projects, the AOPs have been entered into the AOP KB (see 6.9) and are currently undergoing an OECD external review process:

1. Binding of inhibitor to the Complex I of mitochondrial ETC leads to motor deficit of Parkinson's disease (PD)" (in collaboration with EFSA);
2. Inhibition of Na^+/I^- symporter (NIS) decreases thyroid hormone synthesis leading to learning and memory deficit in children;
3. Binding of agonists to N-methyl-D-aspartate receptor (NMDAR) in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive function;

[30] http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm

4. Binding of antagonist to N-methyl-D-aspartate (NMDA) receptors during brain development (synaptogenesis) induces impairment of learning and memory abilities;
5. PPARα activation leading to impaired fertility upon utero exposure in males;
6. PPARγ activation leading to impaired fertility in adult females; and
7. Protein Alkylation leading to Liver Fibrosis.

A workshop held in 2014, co-sponsored and co-organised by the JRC-EURL ECVAM, contributed to the development of guidance on the principles and best practices for constructing AOPs and AOP networks (Villeneuve *et al.*, 2014a; Villeneuve *et al.*, 2014b).

5.7. Data Reporting Template for Intermediate Effects: OHT201

The basic information most current animal tests deliver is how much of a chemical substance has to be applied to a test animal to trigger an adverse effect like cancer, reproductive toxicity, other effects, or simply death. However, animal tests often fail to answer the fundamental question of how a chemical actually acts in the body when it leads to an adverse outcome?

With an answer to this question, science will be able to develop predictive models to foresee the toxicity of a chemical, derived from observations in (non-animal) test systems that happen on molecular, cellular or organ level and long before the actual negative outcome manifests itself in a living organism. Once researchers can capture these observations in a coherent, widely accepted data format (comparable and compatible to the data formats already used today), the results can be used by modellers to build and calibrate their predictive systems, which in turn will be applied to yet untested chemicals without the need to go back to the laboratory.

Such a new data format template has been developed and delivered by the JRC-EURL ECVAM, in collaboration with the OECD and ECHA. The template titled "OHT 201" has now been adopted by OECD as the new standard for reporting "Intermediate Effects", i.e. observations relevant in the explanation and ultimately prediction of toxicity.

OHT 201 will have the following immediate impacts:

- In the upcoming new version of IUCLID 6 (the ICT system used by industry to fulfil reporting obligations under more and more legislative programmes like e.g. REACH), OHT 201 will be part of the selectable reporting endpoints. A public version of IUCLID 6 is foreseen for early 2016.
- The Adverse Outcome Pathway Knowledge Base (AOP-KB) module Intermediate Effects Database (IEDB, see 6.9.) will be implemented by the JRC-EURL ECVAM, using a dedicated IUCLID 6 instance that will be populated by stakeholders submitting filled-in OHT 201 templates (IUCLID export/import files).
- With OHT 201 being implemented in IUCLID, the notion of Intermediate Effects (and implicitly AOPs and predictive toxicology) will get attention in the regulatory world.

This is a first step towards the ultimate goal of replacing animal-test-centred Adverse Outcome observations with alternative-methods-centred Adverse Outcome Pathway considerations as the basis for risk assessment, also in the domain of regulatory decision making.

5.8. Promoting Regulatory Acceptance in the Frame of EMA: JEG3Rs

The European Medicines Agency (EMA) decided in 2010 to establish an expert group, JEG 3Rs[31], 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 3Rs 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 3Rs experts, and representatives from EDQM and the European Commission (e.g. EURL ECVAM). Over the recent years, JEG 3Rs focused on the following areas: a) compliance check of EMA guidelines with 3Rs principles and proposals for revision; b) the development of guidance relating to the acceptance of 3Rs testing approaches[32], c) 3Rs issues related to batch release testing of vaccines, and d) supporting implementation of Directive 2010/63/EU.

5.9. ICH Guideline for the Photosafety Evaluation of Pharmaceuticals

In an effort to recommend international standards for photosafety assessment, and to harmonise such assessments supporting human clinical trials and marketing authorisations for pharmaceuticals, the International Conference on Harmonisation (ICH) has adopted the ICH harmonised tripartite guideline S10 for photosafety evaluation of pharmaceuticals (ICH 2013), which has entered the implementation phase at the regulatory bodies of the European Union (2014), Japan and USA (2015).

According to the S10 guideline, the use of a validated *in vitro* method for the experimental evaluation of phototoxicity should be generally considered in order to reduce the use of animals, following the 3R principles. The S10 guideline provides recommendations for the experimental assessment of the phototoxic and photoallergenic potential of pharmaceuticals given via either the systemic routes or dermal routes and defines the applicability of *in vitro* testing and the conditions when results would trigger further phototoxicity testing in animals.

Validated assays such as the 3T3 NRU-PT and the ROS assay (Onoue *et al.*, 2013) could be used. The applicability of these methods has to be judged case-by-case depending on, for

[31] 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

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

example, the solubility of the active pharmaceutical ingredient or any diluent or vehicle or the application of the relevant UVB dose. Because tissue distribution is usually not a concern for pharmaceuticals given via the dermal route, reconstructed human skin models can be used to determine the phototoxicity potential of clinical formulation. A negative result from a test with reconstructed human skin can serve as an indicator of low phototoxic potential of such clinical formulations.

5.10. VICH Guidelines on Vaccines: VICH Guidelines on Harmonisation of Criteria for Waiving of Target 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 has been working on behalf of EMA with VICH experts on the development of VICH GL50 "Harmonization of criteria to waive the target animal batch safety testing for inactivated vaccines for veterinary use" adopted in 2013 and in force since 1st March 2014.

At present EURL ECVAM is working with experts from the three VICH[33] regions (Europe, Japan, North America) on a comparable VICH guideline for live veterinary vaccines. After several commenting rounds, the guideline is expected to be ready for approval by the VICH[34] Scientific Committee and public consultation.

5.11. Promoting Regulatory Acceptance in the Context of REACH

5.11.1. Update of REACH Annexes to Reflect Scientific Progress: Skin Corrosion/Irritation, Serious Eye Damage/Eye Irritation, Skin Sensitisation and Acute Systemic Toxicity

In recent years, significant progress has been made in the development, validation and regulatory acceptance of alternative test methods for skin irritation/corrosion, serious eye damage/eye irritation, skin sensitisation and acute systemic toxicity. For skin corrosion and irritation, the regulatory accepted alternative methods now allow the generation of data that is adequate for classification and risk assessment for the vast majority of substances, so that *in vivo* testing is only required in exceptional cases. For serious eye damage/eye irritation, there is also a set of regulatory accepted alternative methods, which will in most

[33] International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products; see http://vichsec.org

[34] http://www.vichsec.org/process/the-9-step-procedure.html

cases be sufficient to obtain information that is adequate for classification and risk assessment, either based on the results from a single test or from the combined results of two or more tests. For skin sensitisation, several *in vitro* test methods have been validated and some have already obtained regulatory acceptance. They are expected to be used in the context of IATA, and the decision on the skin sensitisation properties of the substance to be made based on the combined results from test methods and non-testing methods. These alternative methods may allow the assessment of the endpoint of skin sensitisation for many substances based on non-animal tests alone, without the need to resort to *in vivo* testing.

In addition, several reviews and published data have indicated that substances demonstrated to be of low acute toxicity by the oral route are also of low toxicity by the dermal route (e.g. Creton *et al.*, 2010; Seidle *et al.*, 2011, Moore *et al.*, 2013). Building on this evidence, the Acute Toxicity Working Group of the EPAA has identified opportunities to waive animal testing requirements and, in particular, has developed recommendations to waive an acute dermal toxicity study for those substances which are non-toxic via the oral route. EURL ECVAM, by its direct participation in this EPAA acute toxicity project and data analysis, has contributed to drafting these recommendations which were then forwarded to the Commission. As a follow up, in November 2013, the Commission submitted these recommendations to the Competent Authorities for REACH and CLP (CARACAL) for consideration. After several commenting rounds, in July 2014, CARACAL agreed to amend REACH Annex VIII (point 8.5.3) so that substances that have not shown oral acute toxicity up to a limit dose of 2000 mg/kg body weight would not require dermal data[35].

At the end of May 2014, after informal discussions with DG ENV[36] on the necessity to update the REACH Annexes, a request was made to EURL ECVAM to provide an update on the status of replacing *in vivo* testing for skin corrosion/irritation, serious eye damage/eye irritation and skin sensitisation with *in vitro* methods to help DG ENV in understanding if there would be any impact on the current wording in REACH Annexes and need for changes/amendments. Following these initial contacts, DG ENV initiated internal discussions on the need and feasibility to revise the Annexes and, in October 2014, a more in depth technical exchange was initiated with EURL ECVAM, which led to the drafting of a proposal to Member States to amend REACH Annexes VII and VIII concerning skin irritation/corrosion, serious eye damage/eye irritation and skin sensitisation. While the Standard Information Requirements (SIR) for skin irritation/corrosion and serious eye damage/eye irritation in REACH Annex VII is already based on *in vitro* tests, the SIR for these two endpoints on the Annex VIII level is an *in vivo* test. This requirement does not adequately reflect the state of science any more, as the available *in vitro* tests can deliver results that are adequate for classification and risk assessment. In accordance with Article 13(2) of REACH, which requires the European Commission to amend the Annexes of REACH,

[35] http://www.piscltd.org.uk/wp-content/uploads/2014/09/20140714-110033_CA_61_2014-Acute-toxicity-testing-proposal.pdf

[36] European Commission Directorate-General Environment

if relevant, so as to replace, reduce or refine animal testing, a proposal to amend Annexes VII and VIII was therefore put forward by the Commission to allow fulfilling the information requirement for these endpoints based on *in vitro* tests wherever they give information adequate for classification and risk assessment. *In vivo* testing for these endpoints should only be done as a last resort in cases where *in vitro* tests are not able to provide such information. For skin sensitisation, the SIR in Annex VII currently does not make any reference to *in vitro* tests, since such tests were not available at the time the Annexes were established. The Commission has therefore also proposed an amendment to Annex VII for skin sensitisation to explicitly allow the use of *in vitro* test methods as soon as they have reached regulatory acceptance at EU level.

A proposal was presented to the Competent Authorities for REACH and CLP (CARACAL) at its 16th meeting on 10-11 November 2014 for discussion and comments. The CARACAL submitted written comments by the end of 2014, which were discussed at its 17th meeting on 26-27 March 2015. Following CARACAL consultation, the EC prepared a draft Commission Regulation where it proposes:

- For skin irritation/corrosion and serious eye damage/eye irritation, to delete the standard information requirement for an *in vivo* study, which currently applies to substances manufactured or imported at or above 10 tonnes/year, and to replace it by a conditional requirement only applying to substances for which adequate data cannot be generated by *in vitro* testing.
- For skin sensitisation, to introduce an explicit adaptation rule which allows waiving of the *in vivo* study (the current REACH standard information requirement for all substances subject to registration), if the information requirement can be fulfilled by using an alternative testing approach based on the Adverse Outcome Pathway (AOP) concept.
- For acute systemic toxicity, to introduce an adaptation rule which allows waiving the *in vivo* study by the dermal route if the substance does not meet the criteria for classification as acutely toxic or STOT SE (Specific target organ toxicity – single exposure) by the oral route 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.

This draft Commission Regulation is currently undergoing Inter-Service Consultation and is intended to be submitted to the REACH Committee in October 2015.

5.11.2. Update of REACH Guidance on Information Requirements and Chemical Safety Assessment for Skin Corrosion/Irritation, Serious Eye Damage/Eye Irritation and Respiratory Irritation

Section R.7.2 (Skin- and eye irritation/corrosion and respiratory irritation) of Chapter R.7a (endpoint specific guidance) of the REACH Guidance on Information Requirements and

Chemical Safety Assessment recently underwent revision by ECHA (see http://echa.europa.eu/support/guidance/consultation-procedure/closed-reach). EURL ECVAM was invited by ECHA and nominated by DG ENV to participate in a Partner Expert Group (PEG) responsible for reviewing and commenting on ECHA's proposed update to this section of the Guidance.

A PEG meeting was organised at ECHA in November 2014 to discuss the written feedback on the document provided by the PEG members and to further develop the content to ensure that the Guidance is acceptable to all interested parties. ECHA received a total of 580 written comments on the document, of which 158 were provided by EURL ECVAM. Two hundred of the 580 comments were prioritised by ECHA for discussion during the meeting, of which 79 had been provided by EURL ECVAM.

The great majority of EURL ECVAM's comments were accepted by the PEG members and ECHA. These aimed mostly at making sure that 1) the clarity of the document is increased by separating the information on skin corrosion/irritation, serious eye damage/eye irritation and respiratory tract corrosion/irritation into different chapters; 2) 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 skin corrosion/irritation and serious eye damage/eye irritation; 3) the usability of *in vitro* methods as full replacement alternatives is permitted and encouraged where possible; 4) the information about the use of non-testing data is further updated to be aligned with OECD Guidance Document No. 203 on an IATA for skin corrosion and irritation and the latest developments in the area of serious eye damage/eye irritation; 5) the testing and assessment strategies described in the document were revised to become less prescriptive in the order and extent to which the various relevant information sources may be considered.

ECHA used the output of the PEG meeting as the basis for the consolidation of the document. The revised document was provided to the PEG in January 2015 for final proof-reading with the possibility of submitting further comments. The consolidated draft document addressing the new PEG comments was subsequently submitted to the Member State Committee and the Risk Assessment Committee in February 2015 for consultation by written procedure. A final consultation with CARACAL was initiated in April 2015. The final updated Guidance was published by ECHA in July 2015[37].

[37] http://echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf.

5.11.3. Update of REACH Guidance on Information Requirements and Chemical Safety Assessment for Skin and Respiratory Sensitisation

ECHA is currently revising Section R.7.3 (Skin and respiratory sensitisation) of the endpoint specific guidance (Chapter R.7a) of the REACH Guidance on Information Requirements and Chemical Safety Assessment[38].

EURL ECVAM was invited by ECHA and nominated by DG JRC to participate in a PEG responsible for reviewing and commenting on ECHA's proposed updates to this section of the Guidance.

A PEG meeting is foreseen to take place at ECHA in October 2015 to discuss the comments received from the PEG members and to further consolidate the updated guidance.

5.11.4. Collaboration with ECHA

During 2014-2015, the collaboration between the JRC's Institute for Health & Consumer Protection (IHCP) and ECHA continued on a number of fronts related to chemicals. Based on work carried out for ECHA under the terms of a Service Level Agreement, EURL ECVAM published a state-of-the art review of test methods and non-testing (computational) approaches that help promote the 3Rs in the safety assessment of chemicals (Worth *et al.*, 2014). The report reviews the current scientific status of alternatives to animal experiments, such as *in vitro* test methods and computational models, for a range of human health and ecotoxicological endpoints. It describes their availability and applicability based on knowledge of the underlying mechanisms of toxicological actions. In relation to human health, the following endpoints are covered: a) skin irritation and corrosion; b) serious eye damage and eye irritation; c) skin sensitisation; d) acute systemic toxicity; e) repeated dose toxicity; f) genotoxicity and mutagenicity; g) carcinogenicity; h) reproductive toxicity (including effects on development and fertility); i) endocrine disruption relevant to human health; and j) toxicokinetics. In relation to ecotoxicology, the report focuses on methods for acute and chronic fish toxicity.

5.11.5. Chemical Watch and PETA International Science Consortium Webinars

The PETA International Science Consortium, Ltd., and Chemical Watch organised a series of free webinars focusing on alternative methods and testing strategies that can be used to meet REACH requirements. The series ran from October 2014 to June 2015 and consisted of seven webinars, of which four included speakers from EURL ECVAM. The four scientists from EURL ECVAM provided stakeholders and the general public an opportunity to get updated information on alternative methods and testing strategies that could be used to meet REACH information requirements on aspects of toxicological hazard of registered chemicals.

[38] http://echa.europa.eu/support/guidance/consultation-procedure/ongoing-reach;jsessionid=19E2316109FE4C3E2DE8D0C6006743A7.live2

The slides and records of these webinars can be downloaded from the following links:

- http://www.piscltd.org.uk/reaching-alternatives-animal-testing/
- https://chemicalwatch.com/peta-webinars

5.11.5.1. REACH Webinar on Fish Toxicity

EURL ECVAM contributed to the webinar "(Zebra)fish Embryo Acute Toxicity Test to predict short term toxicity to fish (and beyond)". The webinar covered REACH data requirements for short term toxicity testing on fish, OECD acute fish toxicity test (OECD TG 203), the Fish Embryo Acute Toxicity (FET) Test (OECD 236) as a possible alternative to the acute fish toxicity test underpinned by the correlation between the OECD acute fish toxicity test and the fish embryo toxicity test, as well as potential future uses of fish embryos in a much wider array of environmental science and human health applications.

5.11.5.2. REACH Webinar on Acute Systemic Toxicity

The webinar on acute systemic toxicity focused on alternative approaches to mammalian acute systemic toxicity testing. During this webinar, aspects on the EURL ECVAM's strategy to replace, reduce and refine the use of animals in the assessment of acute mammalian systemic toxicity were presented and discussed. The importance of developing adverse outcome pathways (AOPs) and integrated approaches to testing and assessment (IATA) related to acute systemic toxicity was highlighted. Furthermore, improvements in the predictability of the 3T3 NRU assay, development of additional *in vitro* and *in silico* approaches, waiving acute toxicity testing based on e.g., repeated dose data, and refinement of *in vivo* studies, were discussed. This was followed by an overview of industry experiences in using alternative strategies to meet acute systemic toxicity testing requirements.

5.11.5.3. REACH Webinar on Skin Sensitisation

The skin sensitisation webinar focused on alternative methods and testing strategies to meet REACH requirements. In this webinar, the data requirements and *in vivo* classification for skin sensitisation were presented to define what is required to replace the animal test. An overview of the key mechanisms of skin sensitisation, based on the published adverse outcome pathway, and an illustration of the *in chemico* and *in vitro* methods that can be used to assess skin sensitisation with a specific focus on the validated methods was provided. Examples of how these non-animal methods can be combined in testing strategies/integrated approaches were given including how they perform for "real life" substances. Information on the ongoing OECD activity on the development of a guidance document for the reporting of IATA was also provided.

5.11.5.4. REACH Webinar on Serious Eye Damage and Eye Irritation

In the webinar on serious eye damage and eye irritation, the REACH data requirements, the EU CLP/UN GHS classification system, and the drivers of *in vivo* classification for serious eye damage and eye irritation were first discussed in order to define today's requirements to achieve full replacement of the regulatory animal test. The available *in vitro* methods

and how they can be used alone or in combination in testing strategies, such as the top-down or bottom-up approaches, to meet REACH requirements were then described in detail. Information on the ongoing (i) update of the REACH Guidance on Information Requirements and Chemicals Safety Assessment, (ii) update of REACH Annexes VII and VIII, and (iii) development of an OECD Guidance Document on an IATA on serious eye damage/eye irritation were also provided.

5.12. 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.

5.12.1. Waiving of Two-year Carcinogenicity Studies

A review of carcinogenicity testing requirements in different regulatory environments (human and veterinary medicinal products, plant protection products and industrial chemicals) as the main outcome of an EPAA-supported workshop in February 2013 was published in 2014 (Annys *et al.*, 2014). The report discusses the diversity of requirements for carcinogenicity testing across regulatory sectors, and how these studies may be refined to improve hazard evaluation and risk assessment while improving the implementation of the principles of 3Rs.

Building on the outcome of this review, an EPAA activity was launched aiming at the collection of scientific evidence of 3- and 6-months (sub-)chronic studies as predictors of a negative outcome of carcinogenicity bioassays. The University of Wageningen collaborates with the Dutch Medicines Evaluation Board to compile and analyse a database on active pharmaceutical ingredients. The aim is to confirm and expand previous investigations by Sistare *et al.*, (2011), identify opportunities for waiving the two-years carcinogenicity studies based on, *in vitro* genotoxicity testing, and the results of (sub-)chronic toxicity studies. A peer reviewed publication about the project and its outcome is expected for 2016.

5.12.2. Acute Toxicity

In 2013, EPAA started to build a decision framework to replace animals in acute systemic toxicity testing and, whenever animal use cannot be avoided, replace mortality as principal endpoint by clinical signs predictive of mortality at higher dose levels (evident toxicity). The framework also envisages the use of *in vitro* cytotoxicity assays, grouping of chemicals, read-across, QSARs and data from *in vivo* dose range-finding studies to satisfy regulatory requirements for acute systemic toxicity (e.g. REACH). Currently, a data mining exercise focused on the oral and dermal route is underway, which aims at enabling identification of clinical signs predictive of mortality. The work is being carried out in collaboration with the

UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the UK Chemicals Regulation Directorate. Furthermore, in the context of the REACH Regulation and the assessment of acute systemic toxicity for chemicals subject to the 2018 registration deadline, the project team is consulting ECHA in order to ensure that the outcomes of the EPAA project are relevant to the update of ECHA guidance on acute toxicity and classification of chemicals that the Agency is planning to publish in 2016.

A summary of the activities of the project team can be found in the EPAA Activity Report of 2014[39] and additional details in the presentation of Tom Holmes at the 10th EPAA Annual Conference[40], both available from the EPAA website.
In addition, the Acute Toxicity Working Group of the EPAA has developed recommendations to waive an acute dermal toxicity study for those substances which are non-toxic via the oral route. The recommendations were included in a proposal to amend the REACH Annex VIII, which is currently in the adoption process (for more details see section 5.11.1).

5.12.3. Optimised Evaluation of Skin Sensitisation

The third in a series of workshops on skin sensitisation, co-organised by EPAA, CEFIC-LRI and Cosmetics Europe, was hosted by ECHA in April 2015. The workshop aimed at facilitating exchange of information on the current status of alternatives for skin sensitisation testing and assessment among experts from EU member state regulatory agencies, industry, ECHA, OECD, EURL ECVAM and NGOs. Progress in the area has been made since the previous workshop held in 2013 with the adoption at OECD level of two Test Guidelines (TG) on non-animal testing methods (TG 442C: DPRA and TG 442D, ARE-Nrf2 Luciferase Test Method, KeratinoSens™) and a third draft TG on the human Cell Line Activation Test (h-CLAT) in the approval process. Presentations on case studies demonstrating how data from *in silico, in chemico* and *in vitro* methods can be integrated for the purpose of hazard identification and/or potency sub-categorisation set the scene for the workshop discussion. The feasibility of using such approaches in regulatory decision making including their use in a weight-of-evidence approach for fulfilling requirements under the 2018 REACH registration deadline was considered. The workshop participants debated a number of key issues including: how to balance sufficient harmonisation of such approaches for regulatory use whilst guaranteeing a certain level of flexibility to accommodate new methods and methodologies, the level of uncertainties associated with the final prediction that would be tolerated in hazard assessment and to what extent the potency of a sensitiser can be predicted by non-animal methods bearing in mind the limitations associated with the benchmark *in vivo* potency data. It was generally recognised that, despite these challenges, a simple stepwise approach to testing and

[39] https://circabc.europa.eu/sd/a/b71c4155-48fd-47b0-9d5a-3343c75cba6e/ar-2014.pdf
[40] http://ec.europa.eu/growth/sectors/chemicals/epaa/2014_en.htm

assessment involving the use of the validated methods can already be used for substances falling within the applicability of the test methods/approach.

5.12.4. 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.

In a first step, during 2013-2014, key requirements and differences in the various regions were mapped and possible areas for harmonisation defined. A workshop was held on 15 to 16 September 2015 with regulatory bodies and manufacturers discussing findings and possible ways forward.

5.12.5. 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 aiming 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. The project's Technical Committee agreed on four priority vaccines/vaccine groups (diphtheria/tetanus/acellular pertussis vaccines; human rabies vaccines; veterinary rabies vaccines; clostridial vaccines) and established expert working groups to explore ways to implement the consistency approach.

During 2013-2014, two collaborative studies have been carried out. The clostridial vaccines group evaluated cell culture based methods to replace the Minimum Lethal Dose and Total Combining Power assays required for in-process control of *Clostridium septicum* vaccines. This study is carried out in collaboration with the EDQM BSP. The results were discussed at a workshop on 15-16 September 2015 and recommendations will be given on adaptations of the methods to other clostridial vaccines.

The human rabies vaccines group organised a study aiming at identification of the most suitable ELISA for quantitation of glycoprotein-G in rabies vaccines for human use. The overall goal is replacement of the current *in vivo* test for potency testing of rabies vaccines. The European Commission (via EURL ECVAM) is providing funding for carrying out independent statistical data analysis. A workshop took place on 10-11 May 2015 to discuss the findings and make recommendations on further steps, e.g. a formal collaborative study under the umbrella of EDQM BSP or WHO.

5.12.6. EPAA Lead Theme Workshop

In September 2014, the European Partnership for Alternative Approaches to Animal Testing (EPAA) held a workshop with the title "Knowledge sharing to facilitate regulatory decision-making". The workshop brought together representatives from industry, regulators and

scientists (including scientists active in policy advice) to discuss how knowledge sharing involving all relevant actors could improve and accelerate the development, validation, regulatory acceptance and use of alternative methods to animal testing. Four case studies outlined the procedures in place to obtain regulatory acceptance of new test methods in different industry sectors and highlighted potential examples of involvement of various actors in view of facilitating regulatory use of methods. On the background of these examples, the workshop focused on breakout group discussions in view of identifying potential issues that need to be addressed to improve the regulatory acceptance of alternatives to animal testing. The following topics were identified: (1) Networking and communication (including cross-sector collaboration, international cooperation and harmonization); (2) involvement of regulatory agencies from the initial stages of test method development; and (3) improved communication on the necessary prerequisites for test method acceptance including the establishment of specific criteria for regulatory acceptance. Finally, the workshop concluded that data sharing and intellectual property issues may affect many aspects of test method development, validation and regulatory acceptance. The workshop recommended that financial resources are made available to boost education and outreach activities aimed at improving the acceptance and use of alternatives to animal testing.

5.13. Meeting of the European 3Rs Centres

On 21 to 22 April 2015, EURL ECVAM hosted the meeting of twelve 3Rs Centres from across the EU, including EURL ECVAM. The participants shared information on their organisations, presented their activities and explained their views on how to achieve impact in the 3Rs. Specific areas discussed included the identification of priorities to reduce animal use in biomedical research (including addressing concerns related to the increasing use of transgenic animals); communication and dissemination of knowledge and information on the 3Rs; education and training at undergraduate, postgraduate and professional levels; validation of methods towards their use in regulatory safety assessment; and understanding for example how *in vitro* models (e.g. engineered tissue) can be developed and promoted as key enabling biotechnology to stimulate innovation and growth in a variety of sectors. Time was also dedicated to exploring how to define novel 3Rs metrics that could serve to estimate, for example, the impact of 3Rs activities and to identify trends. Key provisions in the Directive 2010/63 on the protection of animals used for scientific purposes were reviewed including the process of project evaluation. A number of opportunities for closer cooperation between centres were identified.

The 3Rs centres agreed to follow up on the priorities that they identified and to pursue their discussions and to meet up again.

6. Dissemination of Information on Alternatives[41]

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

The access to comprehensively described methods is a prerequisite for their use within decision making processes by regulators and scientists or any end-user in biomedical sciences and toxicology. DB-ALM[42] provides standardised descriptions of methods that are at all stages of development, validation or regulatory acceptance in the different policy areas and for different purposes to enhance the knowledge about and uptake of advanced technologies. Information at various level of detail is provided and defined according to pre-determined criteria for data content by experts in the field. Current focus is given to *in vitro* methods used for safety assessments of chemicals and/or formulations, but it is not limited to it.

The method descriptions capture all information elements necessary to allow judgments of its usefulness, that cover: information on the potential of a method including its intended objectives and applications, the scientific principle and need for it, a summary description of study results obtained so far including performance and reliability evaluations as available and appropriate, discussions on strengths and eventual limitations completed with their status of development, validation or regulatory acceptance. Related information of studies performed and chemicals tested are provided as they become available.

6.1.1. Status

Public launch of the revised DB-ALM 2014:

The year 2014 has seen the public launch of the revised database presented at the 9th World Congress on alternatives to animal use in life sciences and received throughout positive comments during online demonstrations. The DB-ALM search interfaces have been entirely redesigned to offer more flexibility during data retrieval based on controlled scientific vocabularies and/or classifications maintained at the JRC and covering key features of advanced and alternative methods. The first release comprised the methods data sector. The remaining data sectors will be subject to subsequent releases.

Contents:

To date the DB-ALM provides the following information:

Information Sector	Data Sheet Number
Topic Summaries	5

[41] The EURL ECVAM databases on alternative methods originate from the Communication of the Commission to Council and European Parliament SEC(91)1794, further reinforced by Directive 2010/63.

[42] DB-ALM: http://ecvam-dbalm.jrc.ec.europa.eu

Method Summaries	163
Protocols	152
Evaluations, EU projects, Validation studies	86
Test Results (individual investigations)	9231
Persons & Institutions active in the field of alternative methods	238
Bibliographic References	7003

During 2014, the online information content has been enhanced leading to the afore listed figures. Priority is placed on methods submitted for validation and on those originating from EU Integrated Projects with focus on methods from SEURAT-1, the largest ever funded research project to develop computational and *in vitro* tools and methods that will underpin new animal-free approaches to the safety assessment which are under definition (see 2.1). The ACuteTox project is still a priority where another eight method summaries and protocols are close to finalisation in collaboration with the respective experts, in addition to those already published. In total, the DB-ALM continues to provide information for *25 topic areas* addressing human health and ecotoxicological effects of chemical substances, mechanistic information, quality control of biological products, and biocompatibility and safety testing of medical devices.

The over 150 biological endpoints (biological processes, responses or effects) measured by the documented methods at various levels of biological organisation comprise:

- interactions on the molecular level (including biochemistry and biokinetics);
- basal cytotoxicity testing;
- functional parameters of organs and tissues; and
- model organism responses.

6.1.2. Usage

The year 2014 has seen a consolidation of the DB-ALM usage maintaining the high level of requests for new subscription reached in 2013 that in total now amounts to 4443 registered users from 82 countries.

The USA and Italy (approx. 10% each), Germany and France (approx. 9% each) and United Kingdom and Spain (approx. 7% each) are the major customers of the DB-ALM, followed by Belgium (approx. 5%), India (approx. 4%), the Netherlands and Brazil (approx. 3%), and Japan, China and Poland with approx. 2% each covering all main user profiles, such as academia, industry, the regulatory community and animal welfare movement.

More and more data retrieval procedures are now done on the revised DB-ALM 2014 version which provides more information, such as methods abstracts, without the need of login. Its use is rapidly growing and the number of visits and the number of consultations of the Methods data sector has more than doubled during the first three months of online access in 2014.

Link to DB-ALM: http://ecvam-dbalm.jrc.ec.europa.eu

6.2. *In silico* Methods: QSAR Model Database

Although thousands of QSAR models have been developed and published in the scientific literature, and some models have been used in regulatory assessment of chemicals in some countries for many years, a transparent validation process and objective determination of the reliability of QSAR models are crucial to further enhance their regulatory acceptance.

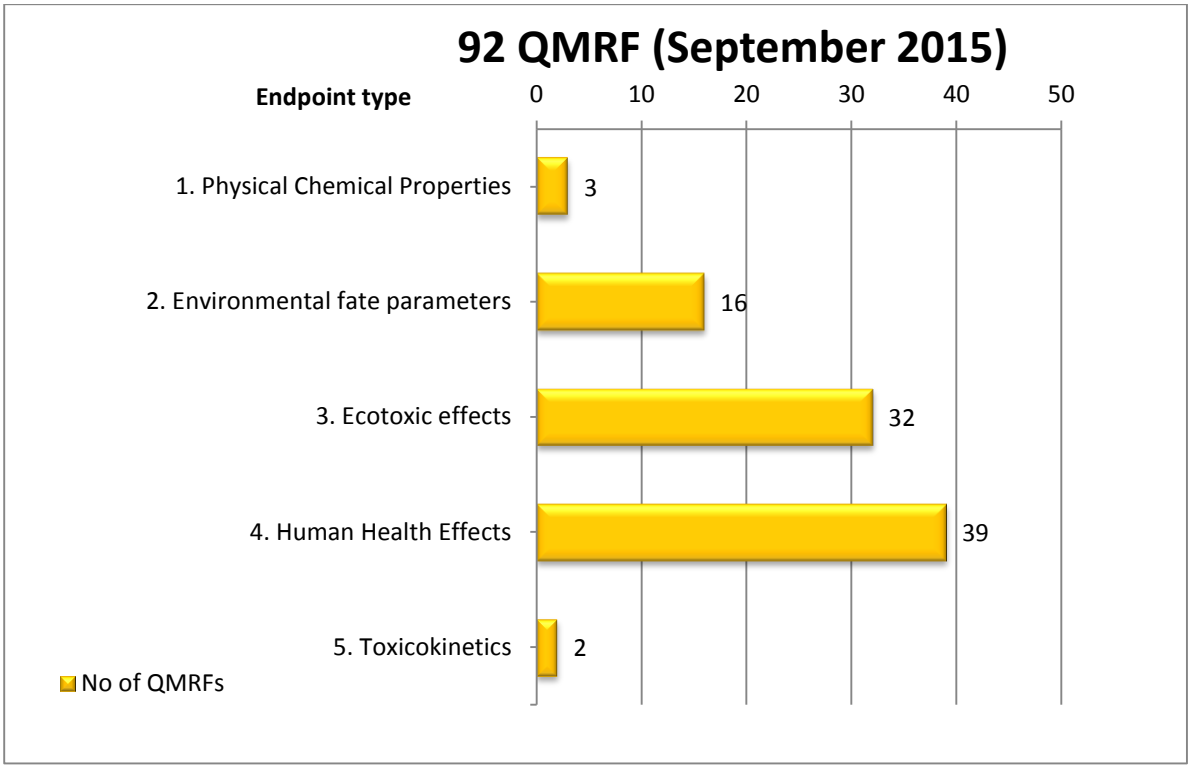
The JRC QSAR Model Database is a freely accessible web application that enables users to submit, publish, and search QSAR Model Reporting Formats (QMRF) reports. Developers and users of QSAR models can submit to the dedicated mailbox information on QSARs by using the QMRF. A downloadable QMRF editor is used for this purpose. The JRC-EURL ECVAM then performs a quality control (i.e. adequacy and completeness of the documentation) of the QMRF submitted. Properly documented QMRFs are included in the JRC QSAR Model Database. Inclusion of the model does not imply acceptance or endorsement by the JRC or the European Commission, and responsibility for use of the models lies with the end-users.

6.2.1. Status

At the time of writing (September 2015), the JRC QSAR Model Database contains 92 QMRFs. Three reports for the endpoint Physical Chemical Properties, 16 reports for Environmental fate parameters, 32 reports for ecotoxic effects, 39 reports for Human Health Effects and two reports for toxicokinetics. Four reports have been published in 2014 and 18 in 2015.

6.2.2 Usage

Overview published reports sorted by endpoint:



As an indication of usage, from 1st to 12th September 2015, the JRC QSAR model Database had a total of 513 visitors (354 unique), averaging 46.1 per day (31.8 unique). Most users in descending order were from Australia, USA, Denmark, China, European Union (i.e., users from the EU institutions), South Korea, Netherlands, Mexico, France and India. The countries extracting the most data are, in descending order, USA, Australia, Denmark, China, European Union, India, South Korea, France, Peru and Spain.
Link to the QSAR Model Database: http://qsardb.jrc.it

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

TSAR[43] 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 on the project during 2014, creating a first beta version that is currently being tested at the EURL ECVAM premises before opening it to the ICATM partners for trial which is planned for the end of this year., After agreement, TSAR will be released to the general public.

[43] TSAR: http://tsar.jrc.ec.europa.eu/index.php?process=1&stage=1

6.4. 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 Europe and is also applied in America, with particular emphasis on South America, used as a resource for higher education in academic institutions and by national authorities for project evaluations. It has now entered the Asian market where it was translated and re-published as a handbook and E-book in Korean.

The EURL ECVAM Search Guide[44] 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.

6.5. Improving Accessibility of Information About Chemicals: "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[45], the chemical information portal launched by EURL ECVAM in 2014, 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 data bases 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 will raise 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 will have a head start when it comes to finding out many details about a chemical.

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

[45] http://chemagora.jrc.ec.europa.eu/chemagora/

6.6. Data about Reference Chemicals for Validating Alternative Methods made accessible: "Chemical List Information System (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[46]) 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.

6.7. EURL ECVAM Genotoxicity and Carcinogenicity Database of Ames Positive Chemicals

The EURL ECVAM Genotoxicity and Carcinogenicity Consolidated database[47] is a structured database that compiles available genotoxicity and carcinogenicity data for 726 Ames positive chemicals originating from different sources. By using a harmonised format to capture the information, this database represents a powerful resource for data analysis that can be used to guide a thorough evaluation of genotoxicity and carcinogenicity.

The generation of such a consolidated database on genotoxicity and carcinogenicity has been demanding in terms of time and effort in order to extract relevant data spread across several databases and to compile them in a harmonised format. Moreover, inconsistencies (e.g. contradictory data derived from different sources) and poor data quality needed to be addressed through rigorous curation which included expert peer review. This investment has led to the public release of a powerful resource for developing and evaluating alternative approaches to animal testing for genotoxicity and carcinogenicity assessment of chemicals.

[46] https://chelist.jrc.ec.europa.eu

[47] https://eurl-ecvam.jrc.ec.europa.eu/databases/genotoxicity-carcinogenicity-db

The database may also serve as a platform for detailed structural characterization of specific groups of compounds with or without carcinogenic or genotoxic activity. The database is linked to two other JRC databases, ChEList and ChemAgora.

The database was constructed following a recommendation of an EURL ECVAM Workshop on 'How can *in vitro* mammalian cell genotoxicity tests reduce the need for *in vivo* follow-up testing with compounds positive in the Ames test?' (Kirkland *et al.*, 2014a).

The key question that drove the construction of the database was whether there is a combination of *in vitro* mammalian cell test results that could complement and mitigate the implications of a positive Ames test response for the prediction of *in vivo* genotoxicity or carcinogenicity. The analysis of the data indicates that in the case of an Ames-positive chemical, negative results in two mammalian cell tests covering both mutation and clastogenicity/aneugenicity endpoints should be considered as indicative of absence of *in vivo* genotoxic or carcinogenic potential (Kirkland *et al.*, 2014b). This analysis has been taken up in the context of genotoxicity evaluation of cosmetic substances within the 9th revision of SCCS's notes of guidance.

6.8. Revision of Reference Lists of Genotoxic and Non-Genotoxic Chemicals

Since their publication in 2008 (Kirkland *et al.*, 2008) EURL ECVAM recommended lists of genotoxic and non-genotoxic chemicals have become the main reference lists for test developers in the area of genotoxicity. Either the whole lists, or parts of them, have been used in the evaluation of a large number of newly developed genotoxicity assays (e.g. Birrel *et al.*, 2010; Mizota *et al.*, 2011; Berthelot-Ricou *et al.*, 2011; Hendriks *et al.*, 2012; Zwart *et al.*, 2012; Rajakrishna *et al.*, 2014; Ireno *et al.*, 2014; Bryce *et al.*, 2014).

The lists have also been employed for the selection of chemicals used to assess whether modifications to existing protocols led to improvements of the assays in terms of performance or served to support the chemical selection of several international inter-laboratory and validation studies of *in vitro* and *in vivo* test methods (Uno *et al.*, 2015; Reus *et al.*, 2013; Dertinger *et al.*, 2012; Sakai *et al.*, 2011; Greywe *et al.*, 2012).

The extensive use of the EURL ECVAM lists of chemicals, as well as the recurring recommendation to refer to them for test method design, development and implementation, prompted EURL ECVAM to revise and update the lists in light of new information (Fowler *et al.*, 2012a, 2014; Kirkland *et al.*, 2014a).

This revised list of chemicals is foreseen to be published by the end of 2015.

6.9. Capturing Adverse Outcome Pathways in a harmonised way: "Adverse Outcome Knowledge Base (AOP-KB) – Wiki"

AOPs are the central element of a toxicological knowledge framework being built to support chemical risk assessment based on mechanistic reasoning (see 5.6). Knowledge that is relevant in the AOP context is not necessarily new, but might already exist. The challenge is that no global effort has yet been undertaken to collect this information. To enable the scientific community to share, develop and discuss their AOP-related knowledge in one central location and using a harmonised structure, the OECD has – in parallel to the instigation of the overall AOP initiative – adopted a first module of its AOP Knowledge Base (AOP-KB[48]), the AOP-KB Wiki. EURL ECVAM and the US EPA jointly developed this tool and are also jointly reponsible for managing and executing the AOP-KB project. In 2014, the first module of the AOP-KB was launched: The AOP-Wiki is a system that organises, via crowd-sourcing, the available knowledge and published research into a verbal description of individual pathways, using 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 ontology-based information.

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.

6.10. Dissemination and Training Activities of EPAA

In the framework of its collaboration with the Institute for *In vitro* Sciences (IIVS), the EPAA supported several initiatives led by IIVS in China to train regulators and scientists to use alternative methods.

In 2014, sponsored by EPAA, IIVS scientists produced a 13-minute video[49] that demonstrates how to perform the Bovine Corneal Opacity and Permeability (BCOP) assay according to OECD Test Guideline no. 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 complete video, with English subtitles, is available at no charge[50]. Translation of subtitles into Chinese and Portuguese is underway and new video versions are expected to be available by the end of 2015.

[48] https://aopkb.org

[49] http://ec.europa.eu/growth/sectors/chemicals/epaa/index_en.htm

[50] Video on YouTube: https://www.youtube.com/watch?v=TiZbp5KDHI8

Other dissemination activities of EPAA comprise the participation in events to raise awareness of alternative approaches among policy makers and parliamentarians. In February 2014, MEP Sidonia Jędrzejewska organised a breakfast meeting on the theme "Animal Testing – Science or Tradition: what futures to alternatives to animal testing?", to which EPAA was invited to give a presentation. In 2014, EPAA was also invited to give a briefing on its activities and structure within a session in the 3-day workshop on animal welfare organised by Eurogroup for Animals and supported by the Intergroup for Animal Welfare of the European Parliament.

7. International Cooperation on Alternative Test Methods (ICATM)

7.1. Background to the EURL ECVAM Activities within ICATM

Regarding the conduct of validation studies, ICATM partners agreed to strengthen their collaboration on a voluntary basis and to create guidance on how validation studies should be conducted. This includes the establishment of validation management groups (or teams VMGs/VMTs) where ICATM members are invited to send experts as liaisons to follow the course of such validation studies. EURL ECVAM is involved in ICATM collaborations as liaisons in VMTs in the areas of eye irritation, skin sensitisation and reproductive and developmental toxicity.

7.2. Collaboration with Japan (JaCVAM): Hand-1 LUC, Vitrigel Tests, IL-8 LUC

Currently, EURL ECVAM activities in VMTs as liaisons include the cooperation in four different validation studies led by JaCVAM i.e., the Hand1-Luc Stem cell test, the Vitrigel-EIT, the Vitrigel-SST and the IL-8 LUC assay for skin sensitisation.

The Hand1-Luc stem cell based test, designed to evaluate developmental toxicity of chemicals is undergoing a JaCVAM-coordinated validation study (LeCoz *et al.*, 2015). Currently, between-laboratory reproducibility and predictive capacity of the test method are assessed. It is anticipated that the Hand1-Luc validation study will be completed by the end of 2015.

The Vitrigel-Eye Irritancy Test (EIT) method is based on a human corneal epithelial cell line, grown on a scaffold of a collagen Vitrigel membrane, containing high-density collagen fibrils equivalent to connective tissue as a three-dimensional cell culture model (Yamaguchi *et al.*, 2013). Changes in the transepithelial electrical resistance (TEER) in response to the exposure of a test chemical, using the barrier function of the epithelium as an indicator allows the estimation of its irritancy potential. In an initial study, 30 chemicals were tested for their correlation between the GHS classification and Vitrigel EIT. A JaCVAM-coordinated validation study according to the conceptual framework of the "modular approach" was launched in 2013 and is still in the experimental phase.

EURL ECVAM is also liaising with JaCVAM on the validation of another Vitrigel-based assay (Vitrigel Skin Sensitisation Test, SST), a test method devising the THP-1-cell line using IL-8 production in response to test chemical incubation for the discrimination between sensitisers and non-sensitisers. The Vitrigel SST validation study was launched in June 2014 and while the experimental phase is still ongoing, the future of this study remains unclear.

EURL ECVAM was also represented, through a nominated expert, on the VMG of the IL-8 Luc assay for skin sensitisation hazard assessment. The test is based on the analysis of the effects of chemicals on the IL-8 promoter activity in THP-1 cells transfected with the IL-8 luciferase reporter gene. The test method was evaluated in a multi-phase validation study coordinated by JaCVAM. The validation exercise aimed at the characterisation of the test method's reliability (transferability, within and between-laboratory reproducibility) and predictive capacity. An independent peer review of the validation study is ongoing. The OECD considered the inclusion of a project proposal for the development of a test guideline on the IL-8 Luc assay method in its work programme in 2015.

7.3. Collaboration with Brazil (BraCVAM): Validation of the Hen's Egg Test on the Chorio-Allantoic Membrane (HET-CAM) for Serious Eye Damage/Eye Irritation in Brazil

The National Institute of Quality Control in Health (INCQS) and the Brazilian Center for Validation of Alternative Methods (BraCVAM) have initiated in 2015 the first ever validation study of an alternative method in Brazil in view of international regulatory acceptance. The study is funded by the Brazilian Ministry of Science, Technology and Innovation (MCTI) and it aims first and foremost at building capacity within Brazil and BraCVAM on the conduct of validation studies of *in vitro* methods for use in a regulatory context. The test method undergoing prospective validation by BraCVAM is the Hen's Egg Test on the Chorio-Allantoic Membrane (HET-CAM) and the main goal of the validation study is to independently assess its reliability (reproducibility within and between laboratories) and relevance (predictive capacity) to identify chemicals not requiring classification for serious eye damage/eye irritation according to UN GHS, combining the experimental results with existing HET-CAM data. This validation study follows the recommendations from ICCVAM (ICCVAM, 2007, 2010) and from the participants of an international workshop on HET-CAM held at the Bundesinstitut für Risikobewertung (BfR) in Berlin, Germany on 29 to 30 October 2012. The validation and regulatory acceptance of HET-CAM for serious eye damage/eye irritation may help further reduce the reliance on animals for this endpoint since HET-CAM has a complementary mechanistic coverage of the endpoint not directly addressed by any of the other available *in vitro* alternatives. HET-CAM may thus play an important role in the IATA for serious eye damage/eye irritation that the JRC-EURL ECVAM and the US EPA will develop for the OECD during the next two years (see 5.3.3.1).

EURL ECVAM is participating in the study as ICATM liason. A first meeting of the validation management group responsible for overseeing and coordinating the validation study was

held in Rio de Janeiro, Brazil on 21-22 May 2015, where various aspects of the validation study were discussed, including: (i) the study objectives and proposed regulatory use of the method, (ii) the study design, (iii) the chemicals selection considering the study objectives and the existing data gaps, and (iv) the test method protocol.

7.4. Collaboration with ICCVAM on ARTAs

EURL ECVAM participates in the ICCVAM Reference Chemical Working group for Androgen Receptor Agonists and Antagonists. This group provides expertise in creating a list of reference chemicals for the evaluation of alternative test methods and testing strategies to identify chemicals that may act as androgen receptor (AR) agonists or antagonists.

8. EURL ECVAM strategies

8.1. EURL ECVAM Strategy to Replace, Reduce and Refine the use of Animals in the Assessment of Acute Mammalian Systemic Toxicity

The EURL ECVAM strategy to replace, reduce and refine the use of animals in the assessment of acute mammalian systemic toxicity[51] was published in December 2014. The strategy underwent consultation with EURL ECVAM's advisory and stakeholder bodies and aims to satisfy regulatory requirements within various pieces of EU legislation. One target is to minimise the use of animals in the assessment of acute systemic toxicity for chemicals subject to the 2018 REACH registration deadline. The strategy will also provide a framework for prioritisation of alternative methods submitted to EURL ECVAM for validation. The implementation of this strategy will rely on the efforts of EURL ECVAM and on the collective and coordinated contribution of a wide range of stakeholders.

In line with the objectives laid down in the strategy, EURL ECVAM has initiated in-house activities aimed at evaluating possibilities to use *in silico* simulation of metabolism in conjunction with the negative result obtained from the 3T3 NRU cytotoxicity assay (predicted oral LD50 > 2000 mg/kg b.w.). Results obtained from the metabolism simulation strategy used (*in silico* metabolite formation followed by prediction of LD50 values for those metabolites) that could allow the confirmation of a negative result or to flag a false negative outcome, are promising. In addition, information regarding the metabolism of the compounds has been collected from databases and the literature and has been used to confirm/exclude metabolism as a reason for false negative predictions and to challenge the outcome of the *in silico* simulations.

Attempts have been made to identify frequent chemical groups in toxic compounds (i.e., oral LD50 ≤ 2000 mg/kg) using the in-house dataset and the Registry of Cytotoxicity. Some chemicals groups were identified as present only and/or mostly in toxic compounds.

[51] https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-strategy-papers/strategy-acute-mammalian-systemic-toxicity

Although these are preliminary results, it is a first step towards the identification of families of chemicals showing acute oral toxicity.

In addition to the lack of metabolic competence of the cell system (3T3 fibroblast), missing toxic effects due to cell-specific toxicity and/or cell-specific functions not captured in these cells, could be another reason for under-classifying acutely oral compounds as non-classified with this *in vitro* cytotoxicity assay. A literature review was started aimed at collecting and organising acute toxic effects by their mechanisms and cell types in eight relevant target organs/systems. Hopefully this will help to improve our mechanistic understanding of acute systemic toxicity, to identify specific mechanisms of toxicity not covered by the 3T3 NRU assay, and to find mechanistic explanations for the identified key structural features. The final aim is to evaluate promising components of integrated approaches for testing and assessment (IATA), including the better use of alternative (*in vitro* and *in silico*) methods.

8.2. EURL ECVAM Strategy to Replace, Reduce and Refine the Use of Fish in Aquatic Toxicity and Bioaccumulation Testing

The assessment of aquatic toxicity and bioaccumulation are important components of the environmental hazard and risk assessment of all types of chemicals and are therefore included in several pieces of EU and international legislations. 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) and vertebrates (fish). Information on bioaccumulation in aquatic organisms is important for understanding the behaviour of a compound in the environment.

Whereas acute aquatic toxicity testing is a basic requirement in most pieces of EU chemicals legislation, chronic aquatic toxicity data may be required when the outcome of the acute testing indicates a risk or when long term exposure is expected. In addition, experimental determination of bioaccumulation may not be necessary if it can be deduced by other means (e.g. consideration of physicochemical properties) that a chemical has a low potential to bioaccumulate.

In this document[52], EURL ECVAM outlines a strategy based on approaches which will deliver an impact on the replacement, reduction and refinement (3Rs) of fish tests used in the assessment of aquatic toxicity and bioaccumulation. The strategy is based on an assessment of the regulatory needs for these endpoints, the scientific state-of-the art and recent activities in these areas. It also intends to provide a framework for the prioritisation of alternative test methods submitted to EURL ECVAM for validation.

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

With regard to aquatic toxicity, the strategy proposes the further development of mechanistically-based replacement alternatives for acute and chronic fish toxicity, as well as the need to revise existing test guidelines to reduce and refine fish testing. Furthermore, the development of guidance on the application of integrated approaches is recommended. This includes the use of data-driven approaches such as interspecies extrapolations, acute-to-chronic relationships and the threshold of toxicological concern approach.

Concerning bioaccumulation, efforts are encouraged for the development and application of *in silico* models such as quantitative structure-activity relationships and physiologically based toxicokinetic models, as well as the standardisation of *in vitro* methods for hepatic metabolism in fish.

EURL ECVAM focuses its own in-house activities on promoting the use of available alternative methods for fish acute toxicity testing, on exploring the usefulness of scientific approaches to support the waiving of chronic fish tests, and on supporting activities at the OECD level.

The achievement of the objectives in pursuit of the strategic aims presented in the strategy document will depend on the proactive and coordinated engagement of multiple stakeholders.

8.3. EURL ECVAM Strategy for achieving 3Rs impact in the assessment of toxicokinetics and systemic toxicity

The EURL ECVAM Strategy document on Toxicokinetics (TK) outlines objectives and activities needed to achieve a 3Rs impact in the area of toxicokinetics and systemic toxicity, with a view to developing a risk assessment approach that is increasingly based on human data[53]. The importance of physiologically-based kinetic (PBK) modelling is a central issue in the strategy document. In order to facilitate the generation, acceptance and use of PBK models in regulatory domains, four main objectives are identified. The first concerns the development of standards to characterise *in vitro* and *in silico* methods that measure individual ADME parameters. The second objective aims to establish good kinetic modelling practices, including a web portal for PBK modelling approaches. The third objective expresses the need for publicly available databases to facilitate access to anatomical and physiological (chemical-independent) information to create PBK models, to store *in vitro* ADME data and human *in vivo* toxicokinetic data. The fourth objective expresses the need to develop guidance on how to generate and use these data in a regulatory setting.

[53] https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/eurl-ecvam-strategy-achieving-3rs-impact-assessment-toxicokinetics-and-systemic-toxicity

8.4. EURL ECVAM Strategy to Replace, Reduce and Refine the Use of Animals in the Assessment of Neurotoxicity

The EURL ECVAM strategy is based on the assessment of the regulatory needs for developmental (DNT) and adult neurotoxicity (ANT) evaluation, as well as the current state of the science in this area, including recent ongoing efforts. Currently, only *in vivo* tests are accepted by regulatory bodies since there are no regulatory approved alternative methods for this purpose. Within the EU legislation of chemical safety assessment, DNT or ANT testing is not a standard requirement, therefore both DNT and ANT evaluation is only performed as higher tiered tests triggered based on structure activity relationships or evidence of neurotoxicity in systemic adult, developmental or reproduction studies.

The existing OECD Test Guideline for developmental (TG 426) and adult neurotoxicity (TG 424) are rarely used due to their complexity, low throughput, high cost and use of a large number of animals. Therefore, new reliable and efficient screening and assessment tools are needed for better identification, prioritisation, and evaluation of chemicals with potential to induce neurotoxicity.

Based on an analysis of the regulatory requirements for DNT and ANT within the relevant pieces of EU legislation, EURL ECVAM has decided to focus efforts on development of an *in vitro* Integrated Testing Strategy (ITS) for neurotoxicity hazard identification. Taking into account current knowledge and requirements for a new paradigm for DNT (Bal-Price *et al.*, 2015a) and ANT testing the proposed ITS will be based on a combination of human cell models and endpoints amenable for high throughput and high content (HT/HC) methods. Such ITS will be based on the flexible integration of test methods that will require explicit specification of context and purpose of use to define confidence required for meeting different regulatory applications. To facilitate the interpretation of the obtained results and to support scientifically defined fit-for-purpose approaches, the applied *in vitro* assays will be anchored to the key events identified in the Adverse Outcome Pathways (AOPs) recently developed for neurotoxicity (Bal-Price *et al.*, 2015b). The AOP concept will be used as a basis for ITS establishment, facilitating use of mechanistic understanding of the pathways leading to toxicity in regulatory decision making.

In addition, EURL ECVAM will continue to support activities aimed at the refinement of relevant *in vivo* studies. The strategy is now under an internal EURL ECVAM's reviewing process. After its final acceptance, the implementation will rely on the cooperation of EURL ECVAM with other related initiatives and the contribution from various stakeholders.

9. Conclusions

During 2014 to 2015, EURL ECVAM continued to further advance the 3Rs through the development and validation of alternative methods for use in regulatory toxicology and through the evaluation, dissemination and promotion of animal-free approaches for use in biomedical research. EURL ECVAM worked on a variety of projects and activities from

research and development projects, evaluation of submitted test methods, validation of alternative methods, promotion of the regulatory acceptance of alternative methods, dissemination, database development, collaboration activities with international partners to the development of EURL ECVAM strategies in specific areas.

Many R&D projects led to significant achievements (e.g., DETECTIVE) thanks to the fruitful collaboration between EURL ECVAM and its partners. These R&D projects cover a broad range of endpoints and adverse effects which are relevant for the protection of human health and the environment. R&D projects in which EURL ECVAM is involved, are investigating the most recent technologies and promising methods, such as e.g. the use of physiologically based toxicokinetics and stem cells technology.

EURL ECVAM evaluated test method submissions which encompass many diverse areas such as fish toxicity, genotoxicity, toxicokinetics, teratogenicity, skin sensitisation, and topical toxicity (skin and eye irritation/corrosion). For each of these, EURL ECVAM consistently evaluated their relevance in terms of the 3Rs, their scientific value, notably considering the mechanistic aspects, performed data analysis, and assessed their possible regulatory uses. EURL ECVAM gave the priority to those test methods addressing regulatory gaps or being the most innovative from a scientific point of view.

EURL ECVAM is involved in several validation studies in collaboration with several partners (e.g., with Cosmetics Europe for the genotoxicity testing) and coordinates the validation study on the AR-CALUX test method that uses EU-NETVAL laboratories for conducting the study for the first time.

EURL ECVAM developed standards for the performance assessment of *in vitro* and *in silico* methods used for the toxicity testing of chemicals which will form the basis of internationally accepted test guidelines and guidance. This also includes the specification of regulatory-targeted reporting standards for describing *in vitro* and *in silico* methods and for recording and communicating data generated with them.

The ESAC reviewed validation studies and issued opinions on test methods in the fields of skin sensitisation, eye and skin irritation and metabolic clearance. Based on these opinions and stakeholder input, EURL ECVAM published two new recommendations.

At the regulatory level, EURL ECVAM developed several (test) guidelines in the frame of its collaboration with several organisations (OECD, ECHA, ICH and VICH). Adopted guidelines are ultimately translated in the relevant European regulations. EURL ECVAM also developed or contributed to the development of several guidance documents related to integrated approaches to testing and assessment (IATA) which guide risk assessors in the use of combined test methods and in the type of conclusions they may draw from these approaches.

EURL ECVAM has also increased its dissemination activities, especially through the development of new databases or the overhaul of existing ones. These databases make available the most recent knowledge on alternative methods to scientists and the public. They also serve as a common tool for EURL ECVAM's partners as they contribute to the consistent use of reliable data.

EURL ECVAM furthermore increased its engagement with a variety of key actors and stakeholders and the 3Rs community as a whole, with the intention of sustaining the levels of commitment and investment on the part of all key players in the field of alternative approaches.

Annex I—Summary status of the adoption of Test Guidelines based on alternative methods in the OECD TG programme (2012-2015)

Table 1 summarises the status of adoption of OECD test guidelines on alternative methods from 2012 to 2015. 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 2012-2015

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)
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 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

Nr.	Toxicity area	Test method description	Acceptance status
			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 irritation	Bovine Corneal Opacity and Permeability (BCOP) test method as included in OECD TG 437/EU TM B.47	Adopted in 2009; updated version (revision of positive controls, use to identify non-classified chemicals and several other revisions) adopted in 2013
7	Serious eye damage/eye	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

Nr.	Toxicity area	Test method description	Acceptance status
	irritation		2013
8	Serious eye damage/eye irritation	Cytosensor Microphysiometer (CM) test method	New draft TG discussed at WNT in 2013 but not yet adopted, pending further clarification on its use to identify non-classified chemicals
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
13	Skin sensitisation	*In vitro* Skin Sensitisation Test: Human Cell Line Activation Test (h-CLAT),	2nd commenting round at OECD finalised. OECD expert group meeting scheduled in October 2015 to discuss final issues.
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)	New draft GD underwent two commenting rounds. Draft GD will be considered for adoption in April 2016
16	Genotoxicity	*In vitro* Mammalian Chromosome Aberration Assay as included in OECD TG 473	Updated OECD TG 473 (originally adopted in 1983)

Nr.	Toxicity area	Test method description	Acceptance status
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
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 (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)
20	Genotoxicity	*In vivo* Mammalian Alkaline Comet Assay	Adopted as new TG 489 in 2014
21	Endocrine disruption	Estrogen receptor transactivation assay (BG1Luc ER TA; agonist and antagonist protocols) as included in OECD TG 457	Adopted in 2012 OECD TG 457 was deleted in 2015. The method was included in OECD TG 455 in 2012 (agonist part) and 2015 (antagonist part) (see table entry below).
22	Endocrine disruption	Performance-Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists	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 was adopted in 2015. The latest update includes the antagonist part of both methods which led to the deletion of OECD TG 457 in parallel as it is no longer needed (see above).
23	Endocrine disruption	Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assays to Detect Chemicals with ER Binding Affinity as included in OECD TG 493	Adopted as new TG in 2015.. It includes two reference test methods: • *In Vitro* Estrogen Receptor (ER) Binding Assay Using a Full Length Human Recombinant ERα; • *In Vitro* Estrogen Receptor Binding Assay Using a Human

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

Annex II—ICATM Alternative Test Methods Validation and Regulatory Acceptance

Table 2. ICATM Alternative Test Methods Validation and Regulatory Acceptance

Method	Current Status	Lead Action Organization	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

Method	Current Status	Lead Action Organization	International Acceptance
			Assessment No. 219), including paragraphs referring to the IATA for Skin Corrosion and Irritation in OECD GD No. 203 and including the use of HPLC/UPLC-spectrophotometry as an alternative procedure to measure tissue viability (increasing the applicability domain of the test methods to coloured substances interfering with the measurement of MTT-formazan)] adopted in 2015
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: EpiDerm™, EpiSkin™, SkinEthic™ RHE**	**Completed**		**OECD TG 439 (2010),** updated version (including the LabCyte™ EPI-model) adopted in 2013 Updated version [deleting the

Method	Current Status	Lead Action Organization	International Acceptance
and LabCyte EPI-MODEL24 *SIT*			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	
***In vitro* test method based on reactive oxygen species (ROS) and**	**Completed**		**ICH S10 (2014)**

Method	Current Status	Lead Action Organization	International Acceptance
photostability			
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, NICEATM-ICCVAM, KoCVAM and Health Canada VMT	
Cytotoxicity test: three-dimensional dermal model (MATREX)	JaCVAM-sponsored validation study in the planning stage	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT	
Cytotoxicity test: Short Time Exposure (STE) test	**Completed**		**OECD TG 491 (2015)**
Use of anaesthetics, analgesics, and	**Completed**		**OECD updated TG 405 (2012)**

Method	Current Status	Lead Action Organization	International Acceptance
humane endpoints for routine use in TG 405			
In vitro approach for categorisation of anti-microbial cleaning products: recommendations for further studies	Completed. EPA/OPP has concluded from submission and review of alternative eye irritation tests conducted on antimicrobial pesticide products with cleaning claims (AMCPs) that the proposed testing approach is acceptable for determining the appropriate eye hazard classification and labelling for AMCPs (see http://www.epa.gov/pesticides/regulating/eye-policy.pdf for the details of the scope of the policy).	NICEATM-ICCVAM	
Cytosensor Microphysiometer® (CM) Test method	The draft TG was submitted to OECD for comments including a set of Performance Standards	EURL ECVAM; NICEATM-ICCVAM	New draft TG discussed at WNT in 2013 but not yet adopted, pending further clarification on its use in a bottom-up approach
Fluorescein Leakage (FL) test method	**Completed**		**OECD TG 460 (2012)**
Human reconstructed tissue models for eye irritation **EpiOcular™ EIT**	**Completed**		**OECD TG 492 (2015)**

Method	Current Status	Lead Action Organization	International Acceptance
Vitrigel-EIT	MAFF-sponsored validation study experimental part finalised	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, Health Canada and KoCVAM VMT liaisons	
Human reconstructed tissue models for eye irritation LabCyte Cornea-model	Performance Standards-based validation study starts in September 2015	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, and KoCVAM liaisons	
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:	KoCVAM validation study is on-going	KoCVAM	

Method	Current Status	Lead Action Organization	International Acceptance
BrdU-Flow Cytometry)			
***In vitro* skin sensitisation assay (DPRA)**	**Completed**		**OECD TG 442C**
In vitro skin sensitisation assay (h-CLAT)	2nd commenting round at OECD finalised for h-CLAT. OECD expert group meeting scheduled in October 2015 to discuss final issues.	EURL ECVAM; JaCVAM and NICEATM-ICCVAM VMT liaison members	
***In vitro* skin sensitisation assay KeratinoSens™**	**Completed**		**OECD TG 442D**
In vitro skin sensitisation assay IL-8 Luc assay	Peer review coordinated by JaCVAM is ongoing	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, KoCVAM and Health Canada VMT liaisons	SPSF to develop a TG approved in 2015
Vitrigel-SST	MAFF-sponsored validation study is pending	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT 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	

Method	Current Status	Lead Action Organization	International Acceptance
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-ICCVAM, 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 - *in vitro* system for deriving information on biotransformation and improving reliability of bio-concentration & bio-accumulation factors (BCF & BAF) and avoiding use of fish bio-concentration tests	Ring trial conducted under the auspices of the OECD is ongoing	United States and European Commission (through JRC-EURL ECVAM)	SPSF for a TG on *in vitro* Fish Hepatic Metabolism approved in April 2014
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:	**Completed**		**OECD TG 457 (2012)** TG 457 has been deleted in parallel to

Method	Current Status	Lead Action Organization	International Acceptance
agonist and antagonist protocols			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-ICCVAM; EURL ECVAM, JaCVAM and KoCVAM VMT liaisons	
Stably transfected CHO Androgen receptor-α transcriptional activation assay for detection of androgenic agonist and antagonist activity of chemicals (AR-STTA)	1st commenting round at OECD finalised for AR-STTA. OECD VMG-NA meeting scheduled in December 2015 to discuss final issues.	JaCVAM and VMG NA liaisons	
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-ICCVAM 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**

Method	Current Status	Lead Action Organization	International Acceptance
Genetic Toxicity Test Methods			
***In vitro mammalian cell* micronucleus test**	**Completed**		**OECD TG 487 (2010), updated TG adopted in 2014**
***In vitro* mammalian cell chromosome aberration assay**	**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-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)	Peer review coordinated by EURL ECVAM completed EURL ECVAM Recommendation published in 2013	JaCVAM ; EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT liaisons	SPSF approved in 2010. New draft OECD GD underwent 2 commenting rounds.
***In vitro* Syrian hamster embryonic cells (SHE) cell transformation assays (CTAs)**	**Completed**		**OECD GD 214 (2015)**
Reproductive Test Methods			
Hand-1 Luc assay	METI-sponsored validation is ongoing	JaCVAM ; EURL ECVAM, NICEATM-ICCVAM, and	

Method	Current Status	Lead Action Organization	International Acceptance
		Health Canada, and KoCVAM VMT liaisons	

References

Aardema MJ, Barnett BC, Khambatta Z, Reisinger K, Ouedraogo-Arras G, Faquet B, Ginestet A-C, Mun GC, Dahl EL, Hewitt NJ, Corvi R, Curren RD, (2010). International prevalidation studies of the EpiDerm™ 3D human reconstructed skin micronucleus (RSMN) assay: Transferability and reproducibility. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **701**, 123–131.

Adriaens E, Barroso J, Eskes C, Hoffmann S, McNamee P, Alépée N, Bessou-Touya S, De Smedt A, De Wever B, Pfannenbecker U, Tailhardat M, Zuang V, (2014). Retrospective analysis of the Draize test for serious eye damage/eye irritation: importance of understanding the *in vivo* endpoints under UN GHS/EU CLP for the development and evaluation of *in vitro* test methods. *Archives of Toxicology* **88**, 701–723.

Al Sharif M, Alov P, Vitcheva V, Pajeva I, Tsakovska I (2014) Modes-of-action related to repeated dose toxicity: from PPARgamma ligand-dependent dysregulation to non-alcoholic fatty liver disease. *PPAR Research, Special issue "PPARs and Metabolic Syndrome",* Volume **2014**, Article ID 432647.

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrano JA, Tietge JE, Villeneuve DL, (2010). Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry* **29**, 730–741.

Annys E, Billington R, Clayton R, Bremm K.-D, Graziano M, McKelvie J, Ragan I, Schwarz M, van der Laan JW, Wood C, Öberg M, Wester P, Woodward KN, (2014). Advancing the 3Rs in regulatory toxicology - Carcinogenicity testing: Scope for harmonisation and advancing the 3Rs in regulated sectors of the European Union. *Regul. Toxicol. Pharmacol*. **69**, 234–242.

Anzali S, Berthold MR, Fioravanzo E, Neagu D, Péry A, Worth AP, Yang C, Cronin MTD & Richarz A-N (2012) Development of computational models for the risk assessment of cosmetic ingredients. *IFSCC Magazine* **15** (4): 249-255. Available at: http://www.cosmostox.eu/publications/printed/

Balls M, Blaauboer BJ, Fentem JH, Bruner L, Combes RD, Ekwall B, Fielder RJ, Guillouzo A, Lewis RW, Lovell DP, Reinhardt CA, Repetto G, Sladowski D, Spielmann H, Zucco F (1995). Practical Aspects of the Validation of Toxicity Test Procedures. The Report and Recommendations of ECVAM Workshop 5. *ATLA* **23**, 129-147.

Bal-Price A, Crofton KM, Leist M, Allen S, Arand M, Buetler T, Delrue N, FitzGerald RE, Hartung T, Heinonen T, Hogberg H, Bennekou SH, Lichtensteiger W, Oggier D, Paparella M, Axelstad M, Piersma A, Rached E, Schilter B, Schmuck G, Stoppini L, Tongiorgi E, Tiramani M, Monnet-Tschudi F, Wilks MF, Ylikomi T, Fritsche E. (2015a). International STakeholder NETwork (ISTNET): creating a developmental neurotoxicity (DNT) testing road map for regulatory purposes. *Arch Toxicol* **89**, 269-87.

Bal-Price A, Crofton KM, Sachana M, Shafer TJ, Behl M, Forsby A, Hargreaves A, Landesmann B, Lein PJ, Louisse J, Monnet-Tschudi F, Paini A, Rolaki A, Schrattenholz A, Suñol C, van Thriel C, Whelan M, Fritsche E, (2015b). Putative adverse outcome pathways relevant to neurotoxicity. *Crit. Rev. Toxicol*. **45**, 83–91.

Bal-Price A, Jennings P, (Eds.), (2014). *In vitro* toxicology systems, *Methods in pharmacology and toxicology*. Humana Press, New York, NY.

Bauch C, Kolle SN, Ramirez T, Eltze T, Fabian E, Mehling A, Teubner W, van Ravenzwaay B, Landsiedel R (2012). Putting the parts together: combining *in vitro* methods to test for skin sensitizing potentials. *Regulatory Toxicology and Pharmacology*; **63**, 489-504.

Belanger SE, Rawlings JM, Carr GJ (2012). An Update to the Fish Embryo Toxicity-Acute Fish Toxicity Relationship and Prospects for Support of the Use of the FET as an Animal Alternative - Document Developed for the February, 2012 Meeting of the OECD ad hoc Expert Group on the Fish Embryo Test and provided to EURL ECVAM as part of the ESAC peer review of the OECD validation study of the Zebrafish Embryo Toxicity Test. Available at: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/files-zvet/Belanger%20et%20al%202012_FET-Fish%20update_OECD%20-1.pdf

Belanger SE, Rawlings JM, Carr GJ (2013). Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. *Environ Toxicol Chem* **32**: 1768-1783

Berggren E. 2014. SEURAT-1 Annual Report, Vol **4**, 86-93. http://www.seurat-1.eu/pages/library/seurat-1-annual-report.php.

Berthelot-Ricou A, Perrin J, Di Giorgio C, De Meo M, Botta A, Courbiere B, (2011). Comet assay on mouse oocytes: an improved technique to evaluate genotoxic risk on female germ cells. *Fertility and Sterility* **95**, 1452–1457.

Birrell L, Cahill P, Hughes C, Tate M, Walmsley RM, (2010). GADD45a-GFP GreenScreen HC assay results for the ECVAM recommended lists of genotoxic and non-genotoxic chemicals for assessment of new genotoxicity tests. Mutation Research/Genetic Toxicology and *Environmental Mutagenesis* **695**, 87–95.

Boobis AR, Doe JE, Heinrich-Hirsch B, Meek ME, Munn S, Ruchirawat M *et al*. (2008). *Crit Rev Toxicol* **38**:87-96.

Braunbeck T, Boettcher M, Hollert H, Kosmehl T, Lammer E, Leist E, Rudolf M, Seitz N, (2005). Towards an alternative for the acute fish LC(50) test in chemical assessment: the fish embryo toxicity test goes multi-species - an update. *ALTEX* **22**, 87–102.

Bryce SM, Bemis JC, Mereness JA, Spellman RA, Moss J, Dickinson D, Schuler MJ, Dertinger SD, (2014). Interpreting *in vitro* micronucleus positive results: Simple biomarker matrix discriminates clastogens, aneugens, and misleading positive agents. *Environ. Mol. Mutagen.* **55**, 542–555.

Busquet F, Strecker R, Rawlings JM, Belanger SE, Braunbeck T, Carr GJ, Cenijn P, Fochtman P, Gourmelon A, Hübler N, Kleensang A, Knöbel M, Kussatz C, Legler J, Lillicrap A, Martínez-

Jerónimo F, Polleichtner C, Rzodeczko H, Salinas E, Schneider KE, Scholz S, van den Brandhof EJ, van der Ven LT, Walter-Rohde S, Weigt S, Witters H, Halder M (2014). OECD validation study to assess intra- and inter-laboratory reproducibility of the zebrafish embryo toxicity test for acute aquatic toxicity testing. *Regulatory Toxicology and Pharmacology* **69**: 496-511.

Ceridono M, Tellner P, Bauer D, Barroso J, Alépée N, *et al.* (2012). The 3T3 neutral red uptake phototoxicity test: practical experience and implications for phototoxicity testing-the report of an ECVAM-EFPIA workshop. *Regul Toxicol Pharmacol*. **63**(3):480-8.

Coecke S, Bowe G, Milcamps A, Bernasconi C, Bostroem A-C, Bories G, Fortaner S, Gineste, J-M, Gouliarmou V, Langezaal I, Liska R, Mendoza E, Morath S, Reina V, Wilk-Zasadna I, Whelan M, (2014). Considerations in the Development of *In vitro* Toxicity Testing Methods Intended for Regulatory Use, in: *In vitro* Toxicology Systems, *Methods in Pharmacology and Toxicology*. pp. 551–569.

Cottrez F, Boitel E, Auriault C, Aeby P, Groux H (2015). Genes specifically modulated in sensitized skins allow the detection of sensitizers in a reconstructed human skin model. Development of the SENS-IS assay. *Toxicology In vitro*. Jun;**29**(4):787-802.

Creton S, Dewhurst IC, Earl LK, Gehen SC, Guest RL, Hotchkiss JA, Indans I, Woolhiser MR, Billington R, (2010). Acute toxicity testing of chemicals-Opportunities to avoid redundant testing and use alternative approaches. *Crit. Rev. Toxicol*. **40**, 50–83.

de Wolf W, Siebel-Sauer A, Lecloux A, Koch V, Holt M, Feijtel T, Comber M, Boeije G (2005). Mode of action and aquatic exposure thresholds of no concern. *Environmental Toxicology and Chemistry* **24**:479-485.

Dertinger SD, Phonethepswath S, Avlasevich SL, Torous DK, Mereness J, Bryce SM, Bemis JC, Bell S, Weller P, MacGregor JT, (2012). Efficient Monitoring of *In vivo* Pig-a Gene Mutation and Chromosomal Damage: Summary of 7 Published Studies and Results From 11 New Reference Compounds. *Toxicological Sciences* **130**, 328–348.

Desprez B, Barroso J, Griesinger C, Kandárová H, Alépée N, Fuchs HW, (2015). Two novel prediction models improve predictions of skin corrosive sub-categories by test methods of OECD Test Guideline No. 431. *Toxicology in Vitro* **29**, 2055–2080.

EC (2008). Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (2008). Available from: http://ec.europa.eu/enterprise/sectors/chemicals/documents/classification/#h2-1

EC EURL ECVAM (2014). Recommendation on the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing. Available at: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations

EC, 2012. Communication from the Commission to the Council "The combination effects from chemicals – Chemical Mixtures" Brussels, 31.05.2012. COM(2012)252 final. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2012:0252:FIN:EN:PDF

ECHA and JRC (2014), "Alternative methods for regulatory toxicology–a state-of-the-art review". Available from: https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/alternative-methods-regulatory-toxicology-state-art-review

ECVAM Performance Standards for In-Vitro Skin Irritation Test Methods based on Reconstructed Human Epidermis, (2009). Available from: https://eurl-ecvam.jrc.ec.europa.eu/validation-regulatory-acceptance/docs-skin-irritation-1/DOC8-updated_ECVAM_PS_2009.pdf

EDQM (2012) Press release of 17 April 2012 "Vaccines for veterinary use adopted by the European Pharmacopoeia Commission at the 142nd Session: International harmonisation with VICH guidelines 41 and 42, deletion of the TABST and 3Rs. Available at: http://www.edqm.eu/medias/fichiers/vaccines_for_veterinary_use_adopted.pdf

EFSA (2012). Scientific Opinion on Exploring options for providing advice about possible human health risks based on the concept of Threshold of Toxicological Concern (TTC). *EFSA Journal* **10**:2750-2853.

EMA (2013) VICH GL50 - Biologicals: harmonization of criteria to waive target animal batch safety testing for inactivated vaccines for veterinary use. EMACVMP/VICH/582610/2009. Committee for Medicinal Products for Veterinary Use (CVMP)

EU (2010). Directive 2010/63/EU of the European Parliament and Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union L* **276**: 33-79

EU (2012). Commission Implementing Decision 2012/707/EU establishing a common format for the submission of the information pursuant to Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. *Official Journal of the European Union L* **320**: 33-50

EURL ECVAM (2013). ESAC Opinion based on the ESAC Peer Review on the validity (reliability and relevance) of the zebrafish embryo toxicity test for acute aquatic toxicity testing. Available as Annex 1 on: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/zfet-recommendation

EURL ECVAM (2014). EURL ECVAM recommendation on the Zebrafish Embryo Acute Toxicity Test Method (ZFET) for Acute Aquatic Toxicity Testing. Available at: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/zfet-recommendation

Fay KA, Fitzsimmons PN, Hoffman AD, Nichols JW (2014). Optimizing the use of rainbow trout hepatocytes for bioaccumulation assessments with fish. *Xenobiotica* **44**:345-51

Fowler P, Smith K, Young J, Jeffrey L, Kirkland D, Pfuhler S, Carmichael P, (2012a). Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. I. Choice of cell type. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **742**, 11–25.

Fowler P, Smith R, Smith K, Young J, Jeffrey L, Carmichael P, Kirkland D, Pfuhler S, (2014). Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. III: Sensitivity of human cell types to known genotoxic agents. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **767**, 28–36.

Fowler P, Smith R, Smith K, Young J, Jeffrey L, Kirkland D, Pfuhler S, Carmichael P, (2012b). Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. II. Importance of accurate toxicity measurement. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **747**, 104–117.

Gajewska M, Paini A, Sala Benito J, Burton J, Worth A, Urani C, Briesen H & Schramm KW (2015c). *In vitro* to *in vivo* correlation of the skin penetration, liver clearance and hepatotoxicity of caffeine. *Food and Chemical Toxicology* **75**, 39-49.

Gajewska M, Paini A, Sala Benito JV, Burton J, Worth A, Urani C, Briesen H, Schramm K-W (2015) *In vitro*-to-*in vivo* correlation of the skin penetration, liver clearance and hepatotoxicity of caffeine. *Food and Chemical Toxicology* **75**: 39-49

Gajewska M, Paini A, Sala Benito JV, Burton J, Worth A, Urani C, Briesen H, Schramm KW. (2015). *In vitro*-to-*in vivo* correlation of the skin penetration, liver clearance and hepatotoxicity of caffeine. *Food Chem Toxicol*. **75**: 39-49.

Gajewska M, Worth A, Urani C, Briesen H & Schramm KW (2014a). Application of physiologically-based toxicokinetic modelling in oral-to-dermal extrapolation of threshold doses of cosmetic ingredients. *Toxicology Letters* **227**, 189-202.

Gajewska M, Worth A, Urani C, Briesen H, Schramm K-W (2014b) The acute effects of daily nicotine intake on heart rate – A toxicokinetic and toxicodynamic modelling study. *Regulatory Toxicology and Pharmacology* **70**(1): 312-324

Gordon VC (1992). The scientific basis of the EYTEX system. ATLA 20, 537-548.

Greywe D, Kreutz J, Banduhn N, Krauledat M, Scheel J, Schroeder KR, Wolf T, Reisinger K, (2012). Applicability and robustness of the hen's egg test for analysis of micronucleus induction (HET-MN): Results from an inter-laboratory trial. Mutation Research/Genetic *Toxicology and Environmental Mutagenesis* **747**, 118–134.

Griesinger C, Desprez B, Coecke S, Casey W, Zuang V "Validation of alternative *in vitro* methods to animal testing: concepts, challenges, processes and tools" (in press, 2015, Springer)

Grinberg M, Stöber RM, Edlund K, Rempel E, Godoy P, Reif R, Widera A, Madjar K, Schmidt-Heck W, Marchan R, Sachinidis A, Spitkovsky D, Hescheler J, Carmo H, Arbo MD, van de Water B, Wink S, Vinken M, Rogiers V, Escher S, Hardy B, Mitic D, Myatt G, Waldmann T, Mardinoglu A, Damm G, Seehofer D, Nüssler A, Weiss TS, Oberemm A, Lampen A, Schaap MM, Luijten M, van Steeg H, Thasler WE, Kleinjans JC, Stierum RH, Leist M, Rahnenführer J, Hengstler JG. 2014. Toxicogenomics directory of chemically exposed human hepatocytes. *Arch Toxicol*. 2014 **88**(12): 2261-87.

Groh KJ, Carvalho RN, Chipman JK, Denslow ND, Halder M, Murphy CA, Roelofs D, Rolaki A, Schirmer K, Watanabe KH (2015b). Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: II. A focus on growth impairment in fish. *Chemosphere* **120**:778-92

Groh KJ, Carvalho RN, Chipman JK, Denslow ND, Halder M, Murphy CA, Roelofs D, Rolaki A, Schirmer K, Watanabe KH. (2015a). Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: I. Challenges and research needs in ecotoxicology. *Chemosphere* **120**:764-77

Gross M, Daginnus K, Deviller G, de Wolf W, Dungey S, Galli C, Gourmelon A, Jacobs M, Matthiessen P, Micheletti C, Nestmann E, Pavan M, Paya-Perez A, Ratte HT, Safford B, Sokull-Klüttgen B, Stock F, Stolzenberg HC, Wheeler J, Willuhn M, Worth A, Comenges JMZ, Crane M (2010). Thresholds of toxicological concern for endocrine active substances in the aquatic environment. *Integrated Environmental Assessment and Management* **6**:2-11.

Gutsell S, Hodges G, Marshall S, Roberts J (2015). Ecotoxicological thresholds - Practical applications in an industrial inventory. *Environmental Toxicology and Chemistry* **34**:935-42

Hastwell PW, Chai L-L, Roberts KJ, Webster TW, Harvey JS, Rees RW, Walmsley RM, (2006). High-specificity and high-sensitivity genotoxicity assessment in a human cell line: validation of the GreenScreen HC GADD45a-GFP genotoxicity assay. *Mutat. Res.* **607**, 160–175.

Hendriks G, Atallah M, Morolli B, Calléja F, Ras-Verloop N, Huijskens I, Raamsman M, van de Water B, Vrieling H, (2012). The ToxTracker Assay: Novel GFP Reporter Systems that Provide Mechanistic Insight into the Genotoxic Properties of Chemicals. *Toxicological Sciences* **125**, 285–298.

ICCVAM (2007). ICCVAM Test Method Evaluation Report: *In vitro* Ocular Toxicity Test Methods for Identifying Severe Irritants and Corrosives. NIH Publication No. 07-4517. National Institute of Environmental Health Sciences, Resesarch Triangle Park, North Carolina, USA. Available from:

http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_tmer.htm

ICCVAM (2010). ICCVAM Test Method Evaluation Report: Current Validation Status of *In vitro* Test Methods Proposed for Identifying Eye Injury Hazard Potential of Chemicals and Products. NIH Publication No. 10-7553. National Institute of Environmental Health Sciences, Resesarch Triangle Park, North Carolina, USA. Available from: http://iccvam.niehs.nih.gov/methods/ocutox/MildMod-TMER.htm

ICH 2009: International Conference on Harmonisation of technical Requirements for Registration of Pharmaceuticals for human use, ICH Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals M3(R2), Step 4 version of 11 June 2009. Available online at (accessed on 26 May 2015): http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M3_R2/Step4/M3_R2__Guideline.pdf

ICH 2013: International Conference on Harmonisation of technical Requirements for Registration of Pharmaceuticals for human use, ICH Harmonised Tripartite Guideline for photosafety evaluation of pharmaceuticals S10, Step 4 version of 13 November 2013. Available online at (accessed on 26 May 2015): http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S10/S10_Step_4.pdf

Ireno I, Baumann C, Stöber R, Hengstler J, Wiesmüller L, (2014). Fluorescence-based recombination assay for sensitive and specific detection of genotoxic carcinogens in human cells. *Arch Toxicol* **88**, 1141–1159.

Isbrucker R, Arciniega J, McFarland R, Chapsal JM, Xing D, Bache C, Nelson S, Costanzo A, Hoonakker M, Castiaux A, Halder M, Casey W, Johnson N, Jones B, Doelling V, Sprankle C, Rinckel L, Stokes W. (2014) Report on the international workshop on alternatives to the murine histamine sensitization test (HIST) for acellular pertussis vaccines: State of the science and the path forward. *Biologicals* **42**:114-122

JaCVAM 2013: Japanese Center for the Validation of Alternative Methods, National Institute of Health Sciences. Peer Review Panel Evaluation of the Reactive Oxygen Species (ROS) Photosafety Assay, October 2013. Available online (accessed on 15 May 2015): http://www.jacvam.jp/files/news/ROS_Peer_Review_Report_FINAL16OCT13.pdf

Jagger C, Tate M, Cahill PA, Hughes C, Knight AW, Billinton N, Walmsley RM, (2009). Assessment of the genotoxicity of S9-generated metabolites using the GreenScreen HC GADD45a-GFP assay. *Mutagenesis* **24**, 35–50.

Johanning K, Hancock G, Escher B, Adekola A, Bernhard MJ, Cowan-Ellsberry C, Domoradzki J, Dyer S, Eickhoff C, Embry M, Erhardt S, Fitzsimmons P, Halder M, Hill J, Holden D, Johnson R, Rutishauser S, Segner H, Schultz I, Nichols J (2012) Assessment of metabolic stability using the rainbow trout (Oncorhynchus mykiss) liver S9 fraction. *Curr Protoc Toxicol. Chapter* **14**:Unit 14.10.1-28.

Johansson H, Albrekt AS, Borrebaek CA, Lindstedt M (2013). The GARD assay for assessment of chemical skin sensitizers. *Toxicology In vitro*, **27**: 1163-1169.

Johansson H, Lindstedt M, Albrekt AS, Borrebaek CA (2011). A genomic biomarker signature can predict skin sensitizers using a cell-based *in vitro* alternative to animal tests. *BMC Genomics* **8**: 12-399.

Kelly CP (1989). EYTEX: an *in vitro* method of predicting ocular safety. *Pharmacopeial Forum* **15**, 4815-5824.

Kienzler A, Berggren E, Bessems J, Bopp S, van der Linden S, Worth A (2014) Assessment of mixtures - Review of regulatory requirements and guidance (EUR 26675 EN)

Kirkland D, Kasper P, Müller L, Corvi R, Speit G, (2008). Recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved

genotoxicity tests: A follow-up to an ECVAM workshop. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **653**, 99–108.

Kirkland D, Zeiger E, Madia F, Corvi R, (2014a). 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? II. Construction and analysis of a consolidated database. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 775–776, 69–80.

Kirkland D, Zeiger E, Madia F, Gooderham N, Kasper P, Lynch A, Morita T, Ouedraogo G, Parra Morte JM, Pfuhler S, Rogiers V, Schulz M, Thybaud V, van Benthem J, Vanparys P, Worth A, Corvi R, (2014b). 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? I. Reports of individual databases presented at an EURL ECVAM Workshop. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 775–776, 55–68.

Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I, Piersma A, Schilter B, Schlatter J, van Schothorst F, Vos JG, Würtzen G (2004). European branch of the International Life Sciences Institute. Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. *Food Chem Toxicol* **42**:65-83.

Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T, (2009). Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test? *Comp. Biochem. Physiol. C Toxicol. Pharmacol*. **149**, 196–209.

Landesmann B, Vinken M. (2013). SEURAT-1 Ann. Rep. 3, 283-321. http://www.seurat-1.eu/pages/library/seurat-1-annual-report.php

Laue H, Gfeller H, Jenner KJ, Nichols JW, Kern S, Natsch A (2014). Predicting the bioconcentration of fragrance ingredients by rainbow trout using measured rates of *in vitro* intrinsic clearance. *Environ Sci Technol* **48**:9486-95

Le Coz F, Suzuki N, Nagahori H, Omori T, Saito K (2015). Hand1-Luc embryonic stem cell test (Hand1-Luc EST): a novel rapid and highly reproducible *in vitro* test for embryotoxicity by measuring cytotoxicity and differentiation toxicity using engineered mouse ES cells. *J Toxicol Sci.***40**(2):251-61.

Mizota T, Ohno K, Yamada T, (2011). Validation of a genotoxicity test based on p53R2 gene expression in human lymphoblastoid cells. *Mutat. Res*. **724**, 76–85.

Moore NP, Andrew DJ, Bjerke DL, Creton S, Dreher D, Holmes T, Prieto P, Seidle T, Rowan TG, (2013). Can acute dermal systemic toxicity tests be replaced with oral tests? A comparison of route-specific systemic toxicity and hazard classifications under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). *Regul. Toxicol. Pharmacol*. **66**, 30–37.

Nagel R, (2002). DarT: The embryo test with the Zebrafish Danio rerio--a general model in ecotoxicology and toxicology. *ALTEX* **19** Suppl 1, 38–48.

Nichols JW, Huggett DB, Arnot JA, Fitzsimmons PN, Cowan-Ellsberry CE (2013). Toward improved models for predicting bioconcentration of well-metabolized compounds byrainbow trout using measured rates of *in vitro* intrinsic clearance. *Environ Toxicol Chem* **32**:1611-22

OECD (1992). Fish, acute toxicity test. OECD Guideline for Testing of Chemicals No. 203, OECD, Paris, France

OECD (2004), Test No. 432: *In vitro* 3T3 NRU Phototoxicity Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing.

OECD (2011). Validation report (Phase 1) for the zebrafish embryo toxicity test. Series on Testing and Assessment No. 157. Available at http://www.oecd.org/env/ehs/testing/48572244.pdf (Part 1) and (Part 2) http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO%282011%2940&doclanguage=en

OECD (2012). Validation report (Phase 2) for the zebrafish embryo toxicity test. Series on Testing and Assessment No. 179. (Summary: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282012%2925&doclanguage=en and annexes

http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282012%2925/ANN&doclanguage=en

OECD (2013). Fish Embryo Acute Toxicity (FET) Test. OECD Guideline for Testing of Chemicals No 236, OECD, Paris, France

OECD (2013). Fish, Early-life Stage Toxicity Test. OECD Guideline for Testing of Chemicals No 210, OECD, Paris, France

OECD 2015. Performance standards for assessment of proposed similar or modified *in vitro* skin sensitisation ARE-NrF2 luciferase test methods. Series on Testing and Assessment N. 213.

OECD Test Guideline No. 439 " *In vitro* Skin Irritation: Reconstructed Human Epidermis Test Method", 2010, rev. 2013 and 2015. Available from: http://www.oecd-ilibrary.org/environment/test-no-439-in-vitro-skin-irritation-reconstructed-human-epidermis-test-method_9789264242845-en

Onoue S, Hosoi K, Wakuri S, Iwase Y, Yamamoto T, Matsuoka N, Nakamura K, Toda T, Takagi H, Osaki N, Matsumoto Y, Kawakami S, Seto Y, Kato M, Yamada S, Ohno Y, Kojima H (2012). Establishment and intra-/inter-laboratory validation of a standard protocol of reactive oxygen species assay for chemical photosafety evaluation. *J Appl Toxicol*. **33**(11):1241-50.. doi: 10.1002/jat.2776.

Onoue S, Igarashi N, Yamada S, Tsuda Y (2008). High-throughput reactive oxygen species (ROS) assay: an enabling technology for screening the phototoxic potential of pharmaceutical substances. *J Pharm Biomed Anal*. **7**;46(1):187-93.

Pasonen-Seppänen S, Suhonen TM, Kirjavainen M, Suihko E, Urtti A, Miettinen M, Hyttinen M, Tammi M, Tammi R, (2001). Vitamin C enhances differentiation of a continuous keratinocyte cell line (REK) into epidermis with normal stratum corneum ultrastructure and functional permeability barrier. *Histochem. Cell Biol*. **116**, 287–297.

Piroird C, Ovigne JM, Rousset F, Martinozzi Teissier S, Gomes C, Cotovio J, Alépée N (2015). The myeloid U937 skin sensitisation (U-SENS) addresses the activation of dendritic cell event in the adverse outcome pathway for skin sensitisation. *Toxicology in vitro*. In press.

Ponec M, Weerheim A, Kempenaar J, Mulder A, Gooris GS, Bouwstra J, Mommaas AM, (1997). The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C. *J. Invest. Dermatol*. **109**, 348–355.

Poumay Y, Dupont F, Marcoux S, Leclercq-Smekens M, Hérin M, Coquette A, (2004). A simple reconstructed human epidermis: preparation of the culture model and utilization in *in vitro* studies. *Arch. Dermatol. Res*. **296**, 203–211.

Prunieras M, Regnier M, Woodley D, (1983). Methods for Cultivation of Keratinocytes with an Air-Liquid Interface. *J Investig Dermatol* **81**, 28s–33s.

Rajakrishna L, Krishnan Unni S, Subbiah M, Sadagopan S, Nair AR, Chandrappa R, Sambasivam G, Sukumaran SK, (2014). Validation of a human cell based high-throughput genotoxicity assay "Anthem"s Genotoxicity screen' using ECVAM recommended lists of genotoxic and non-genotoxic chemicals. *Toxicol In Vitro* **28**, 46–53.

Rathman J, Yang C, Arvidson K, Cronin MT, Enoch S, Hristozov D, Lan Y, Madden JC, Neagu D, Richarz AN, Ridley M, Sacher O, Schwab C, Yang L (2013) Development of a COSMOS Database to support in silico modelling for cosmetics ingredients and related chemicals. *The Toxicologist – A Supplement to Toxicological Sciences* 132: 185.

Reus AA, Reisinger K, Downs TR, Carr GJ, Zeller A, Corvi R, Krul CA, Pfuhler S, (2013). Comet assay in reconstructed 3D human epidermal skin models—investigation of intra- and inter-laboratory reproducibility with coded chemicals. *Mutagenesis* **28**, 709–720.

Sakai A, Sasaki K, Hayashi K, Muramatsu D, Arai S, Endou N, Kuroda S, Poth A, Bohnenberger S, Kunkelmann T, Asakura M, Hirose H, Ishii N, Mizuhashi F, Kasamoto S, Nagai M, Pant K, Bruce SW, Sly JE, Yamazaki S, Umeda M, Tanaka N, (2011). An international validation study of a Bhas 42 cell transformation assay for the prediction of chemical carcinogenicity. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **725**, 57–77.

Scholz S, Fischer S, Gündel U, Küster E, Luckenbach T, Voelker D, (2008). The zebrafish embryo model in environmental risk assessment--applications beyond acute toxicity testing. *Environ Sci Pollut Res Int* **15**, 394–404.

Schulte C, Nagel R, (1994). Testing acute toxicity in the embryo of zebrafish, Brachydanio rerio, as an alternative to the acute fish test: preliminary results *ATLA* **22**, 12–19.

Schwanig M, Nagel M, Duchow K, Krämer B (1997). Elimination of abnormal toxicity test for sera and certain vaccines in the European Pharmacopoeia. *Vaccine* **15**, 1047-1048

Scott L, Eskes C, Hoffman S, Adriaens E, Alépée N, Bufo M, Clothier R, Facchini D, Faller C, Guest R, Hamernik K, Harbell J, Hartung T, Kamp H, Le Varlet B, Meloni M, Mcnamee P, Osborn R, Pape W, Pfannenbecker U, Prinsen M, Seaman C, Spielmann H, Stokes W, Trouba K, Vassallo M, Van den Berghe C, Van Goethem F, Vinardell P, Zuang V (2010). A proposed eye irritation testing strategy to reduce and replace *in vivo* studies using Bottom-up and Top-down approaches. *Toxicol In vitro* **24**, 1-9.

Seidle T, Prieto P, Bulgheroni A, (2011). Examining the regulatory value of multi-route mammalian acute systemic toxicity studies. *ALTEX* **28**, 95–102.

Shalom-Feuerstein R, Serror L, De La Forest Divonne S, Petit I, Aberdam E, Odile Damour O, Vigouroux C, Solomon A, Camargo L, Gagioli C, Itskovitz-Eldor J, Ahmad S and Aberdam D (2012). Pluripotent stem cell model reveals essential roles for miR-450b-5p and miR-184 in embryonic corneal lineage specification. *Stem cells* **30**:898-909

Sharif M Al, Alov P, Vitcheva V, Pajeva I, Tsakovska I, (2014). Modes-of-Action Related to Repeated Dose Toxicity: Tissue-Specific Biological Roles of PPARγ Ligand-Dependent Dysregulation in Nonalcoholic Fatty Liver Disease. *PPAR Research* 2014, 1–13.

Sistare FD, Morton D, Alden C, Christensen J, Keller, D, Jonghe SD, Storer RD, Reddy MV, Kraynak A, Trela B, Bienvenu J-G, Bjurström S, Bosmans V, Brewster D, Colman K, Dominick M, Evans J, Hailey JR, Kinter L, Liu M, Mahrt C, Marien D, Myer J, Perry R, Potenta D, Roth A, Sherratt P, Singer T, Slim R, Soper K, Fransson-Steen R, Stoltz J, Turner O, Turnquist S, van Heerden, M., Woicke, J., DeGeorge, J.J., 2011. An analysis of pharmaceutical experience with decades of rat carcinogenicity testing: support for a proposal to modify current regulatory guidelines. *Toxicol Pathol* **39**, 716–744.

Spielmann H, Lovell WW, Hoelzle E *et al*. (1994). *In vitro* phototoxicity testing. The report and recommendations of ECVAM workshop 2. *Alternatives to Laboratory Animals* (*ATLA*) **22**, 314-348.

Suter-Dick, L., Alves, P.M., Blaauboer, B.J., Bremm, K.-D., Brito, C., Coecke, S., Flick, B., Fowler, P., Hescheler, J., Ingelman-Sundberg, M., Jennings, P., Kelm, J.M., Manou, I., Mistry, P., Moretto, A., Roth, A., Stedman, D., van de Water, B., Beilmann, M., 2015. Stem cell-derived systems in toxicology assessment. *Stem Cells Dev*. **24**, 1284–1296.

Takenouchi O, Miyazawa M, Saito M, Saito K, Ashikaga T, Sakaguchi H (2013). Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with high octanol-water partition coefficients. *Journal of Toxicological Sciences*, **38**: 599-609.

Tanneberger K, Knöbel M, Busser FJ, Sinnige TL, Hermens JL, Schirmer K (2013). Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. *Environmental Science & Technology* **47**: 1110-1119

Tsakovska I, Al Sharif M, Alov P, Diukendjieva A, Fioravanzo E, Cronin MTD, Pajeva I (2014) Molecular modelling study of the PPARγ receptor in relation to the Mode of Action/Adverse Outcome Pathway framework for liver steatosis. *Int. J. Mol. Sci.* **15**(5): 7651-7666.

UN (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fifth revised edition. United Nations, New York and Geneva, 2013. Available from: http://www.unece.org/fileadmin/DAM/trans/danger/publi/ghs/ghs_rev05/English/ST-SG-AC10-30-Rev5e.pdf

Uno Y, Kojima H, Omori T, Corvi R, Honma M, Schechtman LM, Tice RR, Beevers C, Boeck MD, Burlinson B, Hobbs CA, Kitamoto S, Kraynak AR, McNamee J, Nakagawa Y, Pant K, Plappert-Helbig U, Priestley C, Takasawa H, Wada K, Wirnitzer U, Asano N, Escobar PA, Lovell D, Morita T, Nakajima M, Ohno Y, Hayashi M, (2015). JaCVAM-organized international validation study of the *in vivo* rodent alkaline comet assay for detection of genotoxic carcinogens: II. Summary of definitive validation study results. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **786**–788, 45–76.

Villeneuve D, Volz DC, Embry MR, Ankley GT, Belanger SE, Léonard M, Schirmer K, Tanguay R, Truong L, Wehmas L (2014). Investigating alternatives to the fish early-life stage test: A strategy for discovering and annotating adverse outcome pathways for early fish development. *Environmental Toxicology and Chemistry* **33**: 158-169

Villeneuve DL, Crump D, Garcia-Reyero N, Hecker M, Hutchinson TH, LaLone CA, Landesmann B, Lettieri T, Munn S, Nepelska M, Ottinger MA, Vergauwen L, Whelan M, (2014a). Adverse Outcome Pathway (AOP) Development I: Strategies and Principles. *Toxicological Sciences* **142**, 312–320.

Villeneuve DL, Crump D, Garcia-Reyero N, Hecker M, Hutchinson TH, LaLone CA, Landesmann B, Lettieri T, Munn S, Nepelska M, Ottinger MA, Vergauwen L, Whelan M, (2014b). Adverse Outcome Pathway Development II: Best Practices. *Toxicological Sciences* **142**, 321–330.

Vinken M, Landesmann B, Goumenou M, Vinken S, Shah I, Jaeschke H, Willett C, Whelan M, Rogiers V. 2013. Development of an adverse outcome pathway from drug-mediated bile salt export pump inhibition to cholestatic liver injury. *Toxicol Sci.* **136**(1): 97-106.

Whelan M, Schwarz M. (2011). SEURAT-1 Annual Report, Vol 1, 47-51. http://www.seurat-1.eu/pages/library/seurat-1-annual-report.php.

Whelan M, Schwarz M. (2012). SEURAT-1 Annual Report, Vol 2, 7-63. http://www.seurat-1.eu/pages/library/seurat-1-annual-report.php.

Williams ES, Berninger JP, Brooks BW (2011). Application of chemical toxicity distributions to ecotoxicology data requirements under REACH. *Environmental Toxicology and Chemistry* **30**:1943-1954.

Wink S, Hiemstra S, Huppelschoten S, Danen E, Niemeijer M, Hendriks G, Vrieling H, Herpers B, van de Water B. (2014). Quantitative high content imaging of cellular adaptive stress response pathways in toxicity for chemical safety assessment. *Chem Res Toxicol*. **27**(3): 338-55.

Wolf T, Niehaus-Rolf C, Banduhn N, Eschrich D, Scheel J, Luepke N-P, (2008). The hen's egg test for micronucleus induction (HET-MN): novel analyses with a series of well-characterized substances support the further evaluation of the test system. *Mutat. Res.* **650**, 150–164.

Worth A, Barroso JF, Bremer S, Burton J, Casati S, Coecke S, Corvi R,Desprez B,Dumont C, Gouliarmou V, Goumenou M, Graepel R, Griesinger C, Halder ME, Janusch Roi A, Kienzler A, Madia F, Munn S, Nepelska M, Paini A, Price A, Prieto Peraita P, Rolaki A, Schaeffer MW, Triebe J, Whelan M, Wittwehr C & Zuang V (2014). Alternative methods for regulatory toxicology – a state-of-the-art review. JRC report EUR 26797 EN. Publications Office of the European Union, Luxembourg. Available from: http://publications.jrc.ec.europa.eu/repository/

Yamaguchi H, Kojima H, Takezawa T (2013). Vitrigel-eye irritancy test method using HCE-T cells. *Toxicol Sci.* **135**(2):347-55.

Zaldivar Comenges JM, Menecozzi M, Macko P, Rodriguez R, Bouhifd M & Baraibar Fentanes J, (2011). A Biology-Based Dynamic Approach for the Modelling of Toxicity in Cell Assays: Part II: Models for Cell Population Growth and Toxicity. JRC Scientific and Technical Report EUR 24898 EN. Publications Office of the European Union, Luxembourg. Available from: http://publications.jrc.ec.europa.eu/repository/

Zaldívar JM, Mennecozzi M, Marcelino Rodrigues R & Bouhifd M, (2010). A biology-based dynamic approach for the modelling of toxicity in cell-based assays. Part I: Fate modelling. JRC Scientific and Technical Report EUR 24374 EN. Publications Office of the European Union, Luxembourg. Available from: http://publications.jrc.ec.europa.eu/repository/

Zaldívar JM, Wambaugh J & Judson R, (2012). Modeling *In vitro* Cell-Based Assays Experiments: Cell Population Dynamics. In Models of the ecological hierarchy: from molecules to the ecosphere, F Jørgensen & SE Jordán (Eds), pp 51-71. *Developments in Environmental Modelling* **Vol 25**. Elsevier.

Zuang V, Desprez B, Barroso J, Berggren E, Bernasconi C, Bessems J, Casati S, Coecke S, Corvi R, Griesinger C, Halder M, Janusch-Roi A, Landesmann B, Madia F, Milcamps A, Munn S, Price A, Prieto P, Schäffer M, Wittwehr C, Worth A, Whelan M, Institute for Health and Consumer Protection, EURL ECVAM, (2014). EURL ECVAM, status report on the development, validation and regulatory acceptance of alternative methods and approaches (2013-April 2014). Publications Office, Luxembourg.

Zuang V, Tuomainen AM, Amcoff P, Bernasconi C, Bremer S, Casati S, Castello P, Coecke S, Corvi R, Griesinger C, Janusch Roi A, Kirmizidis G, Prieto P, Worth A, Munn S, Berggren E, Whelan M, European Commission, Joint Research Centre, (2013). EURL ECVAM progress report on the development, validation and regulatory acceptance of alternative methods (2010-2013) prepared in the framework of directive 76/768/EEC and regulation (EC) No 1223/2009 on cosmetic products. Publications Office, Luxembourg. Available online at (accessed on 26 May 2015): http://bookshop.europa.eu/en/eurl-ecvam-progress-report-on-the-development-validation-and-regulatory-acceptance-of-alternative-methods-2010-2013--pbLBNA25981/

Zwart EP, Schaap MM, van den Dungen MW, Braakhuis HM, White PA, van Steeg H, van Benthem J, Luijten M, (2012). Proliferating primary hepatocytes from the pUR288 lacZ plasmid mouse are valuable tools for genotoxicity assessment *in vitro*. *Environmental and Molecular Mutagenesis* **53**, 376–383.

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