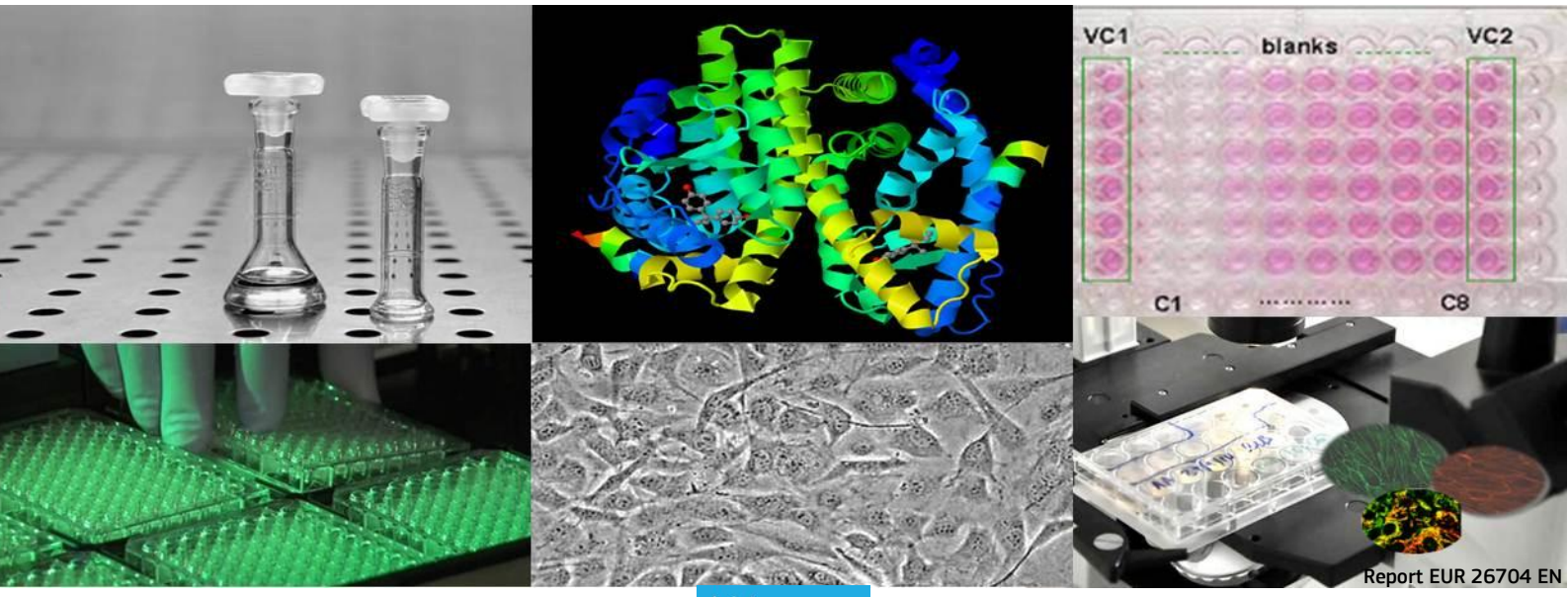
European Commission

JRC SCIENCE AND POLICY REPORTS

EURL ECVAM strategy to replace, reduce and refine the use of animals in the assessment of acute mammalian systemic toxicity

Pilar Prieto, Julien Burton, Rabea Graepel, Anna Price, Maurice Whelan, Andrew Worth

2014



Report EUR 26704 EN

Joint Research Centre

European Commission
Joint Research Centre
Institute for Health and Consumer Protection

Contact information
EURL ECVAM
Address: Joint Research Centre, Via Enrico Fermi 2749, TP 126, 21027 Ispra (Va), Italy
E-mail: JRC-ECVAM-CONTACT@ec.europa.eu

https://ec.europa.eu/jrc/en/institutes/ihcp
https://ec.europa.eu/jrc

JRC90611

EUR 26704 EN

ISBN 978-92-79-38867-5 (PDF)

ISSN 1831-9424 (online)

doi:10.2788/86684

Luxembourg: Publications Office of the European Union, 2014

Abstract
Information on acute systemic toxicity represents a standard requirement within several pieces of chemicals legislation in the EU. One of the main drivers of conducting the test is classification and labelling. Currently, only *in vivo* tests are accepted by regulatory bodies and most of the standard tests use lethality as endpoint. Based on an assessment of the regulatory needs and the scientific state-of-the art in the area, EURL ECVAM considers that efforts should be directed towards a) the reduction and replacement of animal tests for the identification and classification of acute systemic toxicity, and b) the refinement of *in vivo* studies. Consideration should be given to collecting, organising and applying mechanistic knowledge related to this endpoint, to provide a strong mechanistic basis for the design and validation of integrated prediction models. EURL ECVAM proposes to evaluate promising components of integrated approaches for testing and assessment (IATA), including the better use of existing alternative methods, such as mechanistically relevant *in vitro* assays. Information on repeated dose toxicity might also be useful in supporting classification and labelling for acute systemic toxicity. One clear target is minimising animal use for satisfying information requirements for acute systemic toxicity in relation to the 2018 REACH registration deadline. The aims and objectives underpinning the EURL ECVAM strategy can only be achieved through the coordinated and concerted efforts of all stakeholders.

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
Systems Toxicology Unit
EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM strategy to replace, reduce and refine the use of animals in the assessment of acute mammalian systemic toxicity

Executive summary

The assessment of acute systemic toxicity is a component of the safety assessment of substances in the context of EU and international legislation. Information requirements vary depending on the type of substance subject to regulation and the region. In preclinical drug development, however, these studies are no longer required by default to support first clinical trials and their value for overdose and poisoning assessment has been questioned. One of the main drivers for the assessment of acute systemic toxicity is classification and labelling. Currently only data derived from animal tests are accepted by regulatory bodies, which include reduction and refinement methods for the oral and inhalation route. Most of the standard *in vivo* tests use lethality as the endpoint, even though this has been widely criticised both on animal welfare and scientific grounds. Cell-based methods, and in particular *in vitro* cytotoxicity assays, are recognised as additional tests that can be used for estimating the initial doses for tests *in vivo*. However, to date, this approach has not been widely taken up in practice and its contribution to reducing animal numbers has been questioned. The development of mechanistically-based alternative methods and strategies for acute systemic toxicity is hampered by the limited understanding of the key acute toxicity pathways in humans.

This report outlines the strategy proposed by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) for achieving a 3Rs impact in the area of acute systemic toxicity assessment. The EURL ECVAM strategy is based on an assessment of the regulatory needs for this health effect and the scientific state-of-the art in the area. Apart from specifying aims and associated objectives to progress this field, the strategy is also intended to provide a framework for the prioritisation of alternative test methods submitted to EURL ECVAM for validation.

EURL ECVAM considers that efforts in this area should be directed towards the reduction and eventual replacement of animal tests for the identification and classification of acute systemic toxicity. Consideration should be given to collecting and organising mechanistic knowledge related to acute systemic toxicity in order to improve the design and validation of predictive models and approaches such as Integrated Approaches to Testing and Assessment. In this regard, EURL ECVAM proposes to explore scientific options to support the waiving of acute systemic toxicity testing, including the better use of existing alternative methods such as mechanistically relevant *in vitro* assays, as well as existing information on repeated dose toxicity. Efforts should also continue in the refinement of *in vivo* studies when they are necessary. The implementation of this strategy will rely not only on the efforts of EURL ECVAM but on the collective and coordinated contribution of a wide range of stakeholders.

Abbreviations and Glossary Terms

3Rs	Replacement, Reduction, Refinement
ADME	Adsorption, Distribution, Metabolism and Excretion
AOP	Adverse Outcome Pathway
ACuteTox	Optimisation and Prevalidation of an In Vitro Test Strategy for Predicting Human Acute Toxicity
CARACAL	Competent Authorities for REACH and CLP
CLP	Classification, Labelling and Packaging of Substances and Mixture
COSMOS	Integrated *In Silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimase Safety
DNEL	Derived No-Effect Level
EPAA	European Platform for Alternatives to Animal Testing
ECHA	European Chemicals Agency
EU	European Union
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FCP	Fixed Concentration Procedure
FN	False Negative
FP	False Positive
GLP	Good Laboratory Practice
HSI	Humane Society International
IATA	Integrated Approaches to Testing and Assessment
LC_{50}	The concentration to kill 50% of the population (median lethal concentration)
LD_{50}	Single oral/dermal dose to kill 50% of a population (median lethal oral/dermal dose)
NC3Rs	National (UK) Centre for Refinement, Reduction, Replacement
NGO	Non-governmental organisation
NIH	National Institute of Health
NOAEL	No Observed Adverse Effect Level
NRU	Neutral Red Uptake
OECD	Organisation for Economic Co-operation and Development
PBTK	Physiologically-Based Toxicokinetics
QSAR	Quantitative Structure-Activity Relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SEURAT-1	Safety Evaluation Ultimately Replacing Animal Testing – phase 1
TG	Test Guideline
TN	True Negative
TP	True Positive
TK	Toxicokinetics
tpy	Tonnes per year
UN GHS	United Nations Globally Harmonised System of Classification and Labelling
UK	United Kingdom
WNT	OECD Working Group of National Coordinators for the Test Guideline Program

Contents

Executive summary 3
Abbreviations and Glossary Terms 4
Contents 5
1. Introduction 6
2. Strategy to avoid, reduce and refine the use of animals in the assessment of acute systemic toxicity 9
2.1 Strategic Aim 1: Reduction and replacement of animal testing in the assessment of acute systemic toxicity 10
2.2 Strategic Aim 2: Refinement of animal studies for acute systemic toxicity 15
3. Conclusions 17
4. References 18
Annex I Regulatory Requirements for Acute Systemic Toxicity 22

1. Introduction

Acute systemic toxicity comprises the general adverse effects that occur after a single or multiple exposure of an animal to a substance within 24 hours and during an observation period of at least 14 days. The substance may be administered orally, by inhalation or dermally.

The assessment of acute systemic toxicity is one component in the safety evaluation of substances and represents a standard information requirement within several pieces of EU chemicals legislation, including the Regulation on Classification, Labelling and Packaging (CLP) of substances and mixture (EU, 2008a), the Regulation concerning the Registration, Evaluation, Authorisation and Restriction of chemicals (REACH) (EU, 2006), the Biocidal Products Regulation (EU, 2012), the Plant Protection Products Regulation (EU, 2009a) and the Cosmetic Products Regulation (EU, 2009b). Currently, only *in vivo* tests are accepted by regulatory bodies. However, *in vivo* acute systemic toxicity studies are prohibited for cosmetic substances and products (EC, 2009b). Following the provisions of the REACH Regulation and its Annex XI, weight of evidence, qualitative or quantitative structure-activity relationship (QSAR) models, information from structurally related substances (grouping or read-across), and *in vitro* tests can be proposed by the Registrant instead of standard *in vivo* data, provided that adequate documentation and coverage of the standard parameters and observations are included in the dossier submitted for evaluation to the European Chemicals Agency (ECHA).

In preclinical drug development, these studies are no longer required by default to support first clinical trials in man. In many circumstances, the information needed can be obtained from other tests that use non-lethal endpoints and that are already carried out as part of the drug development process. Further, their value for Phase III pharmacological overdose and poisoning assessment has been questioned (Robinson et al., 2008; ICH, 2009; Chapman et al., 2010). Information on data requirements according to the different regulations is included in Annex I.

For the oral route the *in vivo* studies include three refinement and reduction methods described in OECD test guideline (TG) 420 (fixed dose procedure) and EU Test method B.1 bis, OECD TG423 (acute toxic class method) and EU Test method B.1 tris, and OECD TG425 (up and down procedure) (OECD, 2001a, b, c; EU, 2008b). For acute dermal toxicity, the only guideline available is the classical dermal LD_{50} study (TG402, OECD, 1987a; EU Test Method B.3, EU, 2008b). For inhalation toxicity there is a revised version of the classical LC_{50} study (TG403, OECD, 2009a; EU Test Method B.2, EU, 2014) and the acute toxic class method (TG436, OECD, 2009b; EU Test Method B.52; EU, 2014). Moreover, TG420, TG423, TG425 and TG436 use the fewest animals.

The endpoint measured in the majority of these standard assays is animal morbidity or death while evident signs of toxicity (clear signs of toxicity indicate that exposure to the next highest concentration would cause severe toxicity in most animals within the observation period) is only used in the oral fixed dose procedure. The use of lethality as an endpoint has

long been criticised on animal welfare grounds, while the utility of the actual data generated by acute toxicity tests with regard to their ultimate purpose, namely to predict the human hazard potential of substances, has also been questioned. Moreover, Directive 2010/63/EU on the protection of animals used for scientific purposes states under point recital (14):

"The methods selected should avoid, as far as possible, death as an end-point due to the severe suffering experienced during the period before death. Where possible, it should be substituted by more humane end-points using clinical signs that determine the impending death, thereby allowing the animal to be killed without any further suffering."

One of the main purposes of conducting these *in vivo* tests is to categorise substances according to their potential hazard, the dose required to cause toxicity, and to communicate specific information on the hazard concern to workers, emergency responders and consumers (i.e. to support regulatory classification and labelling decisions). This has been confirmed by surveys carried out by the pharmaceutical industry (Robinson et al., 2008) and by the European Partnership for Alternative Approaches to Animal (EPAA) covering other sectors (Seidle et al, 2010).

The currently applied classification systems are based on arbitrary cut-off values for LD_{50} which are then used to estimate human acute toxicity. Within the EU, the CLP Regulation is used to classify chemicals on the basis of acute oral toxicity into four toxicity categories (categories 1 to 4 of the United Nations Globally Harmonised System of Classification and Labelling - UN GHS, see tables 1 and 2 in Annex I). CLP itself does not set information requirements for health hazards and thus classification may be carried out on the basis of available information (EU, 2008a).

Additional scientific drivers for conducting these studies, such as dose setting for repeated dose studies, can be obtained from other study types such as the dose escalation studies carried out to identify maximum tolerated dose (Zbinden and Flury-Roversi, 1981; Robinson et al, 2008; Seidel et al, 2010).

Human reference values such as the acute reference dose, the acceptable daily intake and acute systemic Derived No-Effect levels (DNELs) for risk assessment are usually derived from repeat dose studies (see Annex I, section 1.2 and table 3). Acute human poisoning is treated on the basis of actual clinical symptoms rather than rat LD_{50} values. Only if no other data for systemic toxicity are available then acute systemic toxicity data may be useful for classification/labelling based risk mitigation measures (e.g. setting of occupational exposure limits and chemical emergency response planning).

Acute systemic toxicity after oral, dermal or inhalation exposure requires that the substance becomes bioavailable to a certain extent at the target site. This means that kinetic factors, and importantly absorption, are key determinants of toxicity as indicated in the EURL ECVAM strategy report on toxicokinetics (Bessems et al, in preparation). In addition, if the damage involves interference with homeostatic mechanisms, non-exposed tissues and vital organs can also be affected. For example, respiratory depression leading to death may be due to

depression of the central nervous system rather than a direct effect on the respiratory system (Gennari et al., 2004).

Basal cytotoxicity is certainly a key event in many prevalent toxicological modes-of-action associated with acute health effects. It covers many general mechanisms of toxicity common to most cell types that can lead to organ failure, including for example, disruption of membrane structure or function, inhibition of mitochondrial function, disturbance of protein turnover, and disruption of metabolism and energy production. The mechanisms involved in cytotoxicity and susceptible functions compromised in organ failure have been discussed in numerous papers (Gennari et al, 2004; NIH, 2009; Hartung, 2008, 2014).

The possibility to use cell-based methods to predict acute oral toxicity has been extensively investigated. In this regard, *in vitro* cytotoxicity assays have been developed and evaluated against *in vivo* oral LD_{50} data (correlative approaches) within the context of several international projects (Ekwall, 1999; Halle, 2003; NIH, 2006, Prieto et al., 2013a, b). To date cytotoxicity assays have been considered only as additional tests that can be used for estimating the initial doses for acute oral systemic toxicity tests *in vivo* (OECD, 2010). It appears, however, that this approach has not been widely taken up in practice. The usefulness of the 3T3 NRU assay for predicting the *in vivo* classification and for predicting the starting dose for the subsequent *in vivo* test was also evaluated by Schrage et al. (2011). Their analysis demonstrated a low overall accuracy of the 3T3 NRU in predicting the acute oral toxicity categories (NIH, 2006) and on this basis, the authors questioned its contribution to reducing animal numbers when used to estimate the starting dose for the animal test.

Nevertheless, the evidence also indicates that the 3T3 NRU basal cytotoxicity assay can be used to support the identification of negatives (non-classified substances), with the caveat that due to the limitations of this test method, results should always be used in combination with other information sources to build confidence in the decision not to classify a substance for acute oral toxicity. As stated in a recent EURL ECVAM Recommendation, the applicability domain of the 3T3 NRU needs further characterisation (EC-EURL ECVAM, 2013).

In addition to using basal cytotoxicity, it will also be important to identify cell types and *in vitro* endpoints that are indicative of cell-type specific toxicities, with a view to integrating such endpoints into Integrated Approaches to Testing and Assessment (IATA). For instance, in safety pharmacology studies, the cardiovascular, respiratory and central nervous systems are assessed in a core battery since they are considered vital organs or systems, the functions of which are acutely critical for life (ICH, 2000). Thus the information provided by combinations of relevant *in vitro* assays is expected to have an important contribution in future IATA.

The purpose of this document is to present the EURL ECVAM strategy to avoid, reduce and refine animal testing for acute toxicity hazard identification and classification. The focus is on acute effects that are systemic in nature and, therefore, local effects are not covered. The ultimate aim is to propose solutions that can satisfy information requirements under several

pieces of EU legislation and that can also be considered by the OECD in the context of globally harmonised approaches for the assessment of acute systemic toxicity.

The strategy is intended to be inclusive and as such, its implementation will rely on cooperation between EURL ECVAM and various stakeholders and the coordination of complementary initiatives addressing the strategic aims and related objectives outlined here.

2. Strategy to avoid, reduce and refine the use of animals in the assessment of acute systemic toxicity

In those industrial sectors in which acute systemic toxicity testing is legally required, the data are primarily used to support regulatory decisions on classification and hazard labelling[1]. Therefore, EURL ECVAM proposes that efforts should be directed towards the reduction and eventual elimination of animal tests for the identification and classification of acute systemic toxicity toxicants.

The following two key aims are proposed:

Strategic Aim 1: Reduction and replacement of animal testing in the assessment of acute systemic toxicity

Strategic Aim 2: Refinement of animal studies

The objectives and related activities summarised in Figure 1 have been identified as being necessary to achieve these aims, the realisation of which is expected to have a significant impact on regulatory testing in different industrial sectors.

[1] For extremely hazardous (US) or dangerous (EU) substances the data can be used to derive acute DNELs or acute exposure guideline levels (AEGLs) if exposure occurs via accident or contaminated land.

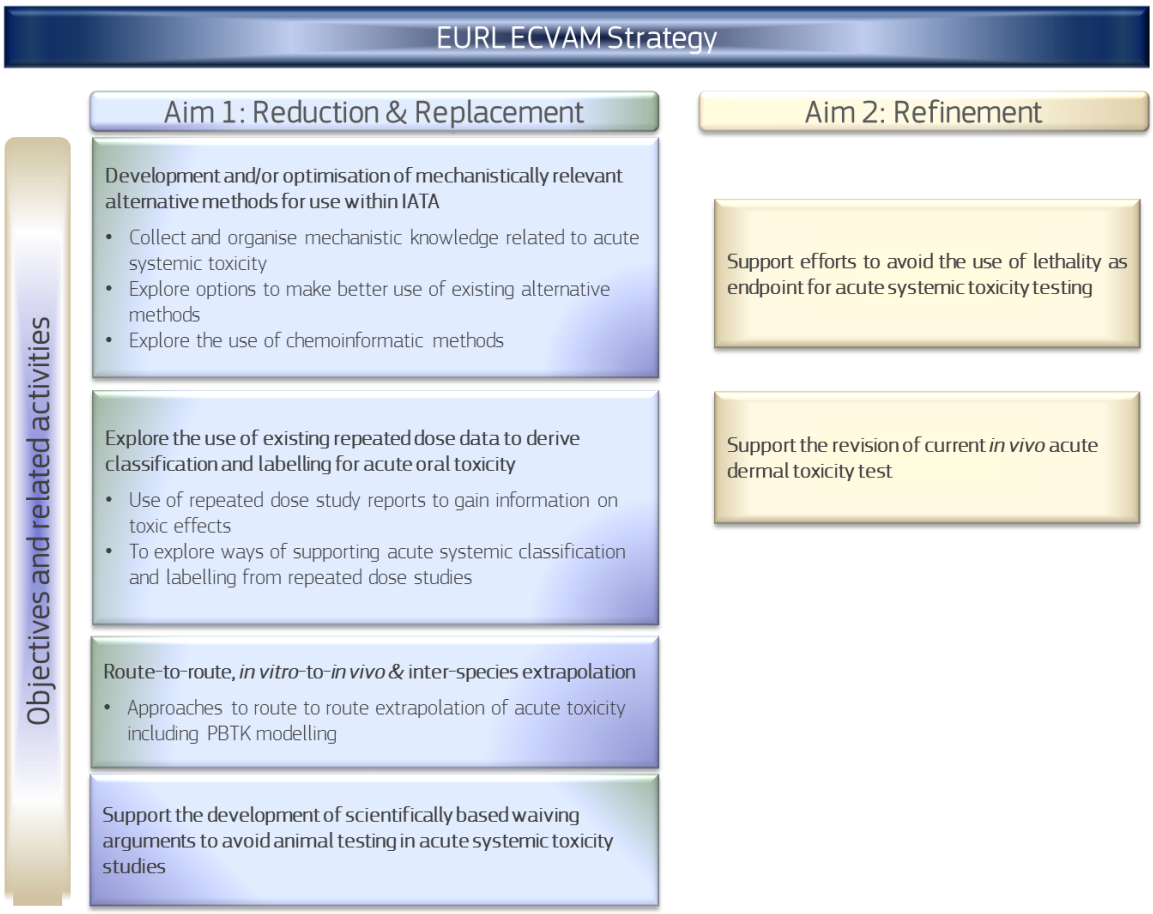


Figure 1. EURL ECVAM strategy to avoid, reduce and refine animal use in the assessment of acute toxicity (IATA - Integrated Approaches to Testing and Assessment; PBTK - Physiologically-Based Toxicokinetics).

2.1 Strategic Aim 1: Reduction and replacement of animal testing in the assessment of acute systemic toxicity

The development of IATA for hazard-based classification is expected to have an impact in terms of replacement and reduction of animal testing. However, as noted above, the development of IATA in this area is hampered by the lack of sufficient mechanistic understanding of the numerous toxicity pathways and/or modes-of-action that lead to acute systemic toxicity. An important consideration is then to improve the theoretical understanding of acute systemic toxicity since this would provide a strong mechanistic basis for the design and validation of integrated prediction models.

The EU FP6 ACuteTox project aimed to develop a non-animal testing strategy for predicting human acute oral toxicity by evaluating and combining cytotoxicity assays, organ-specific toxicity assays, and biokinetic/metabolism methods (Kinsner-Ovaskainen et al., 2013). The project showed the added value of combining the prediction results gained from *in vitro* cytotoxicity assays with information on target organ alerts identified by specific *in vitro*

assays (e.g. neurotoxicity), which helped reduce the number of under-predictions generated by the cytotoxicity assay alone (Prieto et al., 2013a; Zurich et al., 2013).

There are several ongoing activities that, although not directly focused on acute systemic toxicity, could contribute to our understanding of toxicological modes-of-action and also provide innovative methodologies and tools for acute toxicity testing. These are the EU FP7 SEURAT-1 ('Safety Evaluation Ultimately Replacing Animal Testing' - first phase) research initiative (http://www.seurat-1.eu/), focused on alternatives for repeat dose toxicity testing, the Tox21 programme (http://www.epa.gov/ncct/Tox21/), the ToxCast screening programme (http://www.epa.gov/ncct/toxcast/) and the work undertaken by the Hamner Institute for Chemical Safety Sciences (http://www.thehamner.org/institutes-centers/institute-for-chemical-safetysciences/) in the USA.

The gathering and targeted generation of mechanistic knowledge related to systemic toxicity should remain a continuous endeavour within the toxicological community. However, the impact of this effort can be enhanced through the adoption of a more systematic and structured approach to the integration, curation and reporting of such knowledge through the use of the OECD's Adverse Outcome Pathway (AOP) framework and related guidance (OECD, 2013). Provision of this knowledge through the AOP Knowledge Base (www.aopkb.org) will benefit both scientific and regulatory communities in the development, validation and eventual acceptance of alternative approaches for assessing acute systemic health effects.

Taking a fundamentally knowledge-driven approach which is both inspired and supported by empirical evidence, EURL ECVAM proposes the pursuit of the following objectives within this strategic aim:

Objective 1.1. Development and optimisation of mechanistically relevant alternative methods for use within IATA

EURL ECVAM proposes to explore options for making better use of existing *in vitro* and *in silico* methods by investing in the systematic and comprehensive characterisation of their predictive value, possible limitations and applicability domain.

Following on from experience gained during the validation of the 3T3 NRU assay (EC-EURL ECVAM, 2013), the ability to rationalise true/false predictions generated by cytotoxicity assays and to complement the test results with other types of relevant information (e.g. absorption, distribution, metabolism, and excretion (ADME)/toxicokinetics (TK) properties or association with selective mechanisms-of-action that cause organ-specific effects) would provide the basis for a more accurate identification of positive and negative chemicals compared with the use of cytotoxicity assays alone. An example of a tiered assessment approach based on such rationale is illustrated in Figure 2. A negative prediction from the 3T3 NRU cytotoxicity assay (i.e. estimated oral LD_{50} value above the threshold limit of 2000 mg/kg of body weight) would need to be supported with evidence of an absence of bioactivation of the compound, by excluding specific modes-of-action indicative of acute organ-specific toxicity and/or by excluding *in vitro* kinetic processes (e.g. evaporation,

absorption to the plastic, or binding to proteins present in the medium) that could significantly influence the effective (free) concentrations tested. Predicting the ADME properties of a compound in the body could also help identify false positive results derived from *in vitro* cytotoxicity data, due for example to limited *in vivo* absorption or rapid elimination of the compound (metabolism if it is deactivating and excretion). In the validation of such assessment approaches however, misclassifications related to predictions using alternative methods should be interpreted in the light of the imprecision of the *in vivo* method (Hoffmann et al., 2010).

The use of chemoinformatic methods should be explored to identify structural features associated with specific effects at molecular, cellular and tissue levels. It is anticipated that this information will be used in supporting grouping and read-across, guiding cell-specific *in vitro* testing, and complementing the use of *in vitro* cytotoxicity assays.

With a view to supporting such efforts, EURL ECVAM will use the 3T3 NRU cytotoxicity dataset available in-house as a starting point. However, consideration will be given to expand the dataset with under-represented chemical use-categories (e.g. biocides, agrochemicals) and toxicity categories (i.e. GHS category 1 and category 2). The intention is to make the dataset publicly available in order to allow complementary investigations by stakeholders, for example, to evaluate other promising *in vitro* assays to understand their applicability domain and the value of information derived from them.

Based on these efforts, it will be helpful to develop practical guidance, with illustrative case studies, on how to optimally combine *in vitro* assays with other information sources within IATA for the purposes of hazard identification. This will be relevant for the REACH 2018 registration deadline and in particular for substances imported or produced at 1-10 tonnes per year (REACH Annex VII) for which information from acute oral toxicity studies is required but data on repeated dose toxicity may be lacking (see Objective 1.2 below). Under this low tonnage band, information on repeated dose toxicity is not required and therefore often unavailable (see Annex 1, section 1.2). Because the greatest number of substances is expected to be registered for this deadline and a testing proposal for an acute toxicity study is not required, development of this guidance is urgently needed. Such guidance would also be very valuable for the implementation of the EU Regulations on CLP, on Biocidal Products and on Plant Protection Products.

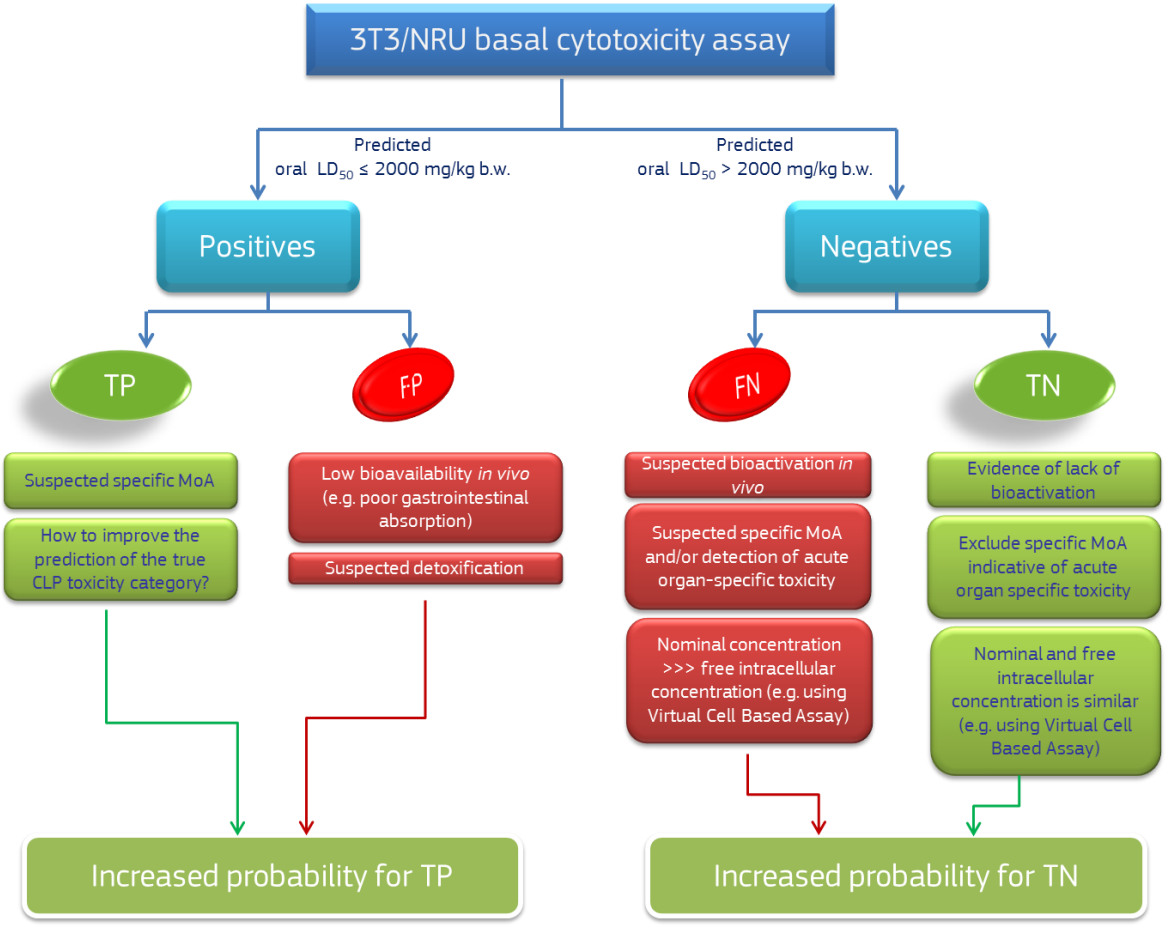


Figure 2. Possible outcomes of the 3T3 NRU cytotoxicity assay when used to identify chemicals that are not classified on the basis of acute oral toxicity (TP = true positive result; FP = false positive result; FN = false negative result; TN = true negative result).

Objective 1.2. Explore the use of repeated dose studies to support classification and labelling for acute oral systemic toxicity

Information on repeated dose toxicity, if available, might be very useful for inferring acute effects. In-house preliminary work by Bulgheroni and colleagues (2009) evaluated the possibility to identify non-classified substances (i.e. those with an oral LD_{50} > 2000 mg/kg) from the results of 28-day repeated dose studies. The findings showed that a 28-day NOAEL threshold of 200 mg/kg b.w. allowed the correct identification of 63% (913/1436) of the non-toxic substances considered.

Building on this work, EURL ECVAM is exploring the use of data from repeated dose studies as a means of gaining information on toxic effects and supporting classification and labelling for acute oral toxicity. In order to obtain input from multiple sectors, EURL ECVAM has launched a survey aimed at gathering additional information and expert opinion in the field of acute systemic toxicity testing and in particular regarding the possibility to conclude on

acute systemic toxic effects from systemic repeated dose studies. The questionnaire has been sent to experts from authorities, academia, industry, NGO's and was publically accessible via the EU survey website until the end of November 2014.

The compilation of a more extensive database of acute and repeated dose study results using reliable information from several available sources would be very beneficial. Analysing such data would be highly relevant for the REACH 2018 registration deadline, in particular for substances imported or produced at 10-100 tonnes per year (Annex VIII) for which repeated dose 28 day study data are also required (see Annex 1, section 1.2). Likewise, waiving acute toxicity tests based on the interpretation of repeated dose data for satisfying information requirements regarding acute effects will be relevant for the classification of active substances used in biocides and plant protection products.

Objective 1.3. Route-to-route, *in vitro* to *in vivo* and inter-species extrapolation

Route-to-route extrapolation offers a solid opportunity to reduce animal testing. Extrapolation from *in vivo* oral toxicity to dermal toxicity is generally expected to be protective (Moore et al., 2013) and thus questions the necessity to test for acute dermal toxicity when oral data already exists. Extrapolation from the oral to the inhalational route has been investigated but is less well established (Seidle et al, 2011).

As part of its involvement in the SEURAT-1 initiative (and in particular the COSMOS project), EURL ECVAM is involved in work aimed at exploring the use of *in silico* models in route-to-route (Gajewska et al, 2014a,b) and *in vitro* to *in vivo* extrapolations (Pery et al., 2013).

Regarding the possibility for inter-species extrapolation and the importance of toxicokinetics, Scholz et al (2014) have recently evaluated the possibility to use the LC_{50s} of the fish embryo acute toxicity test to predict acute mammalian toxicity categories. In their analysis, they took into consideration the impact of species sensitivity, protocol differences and the chorion as potential source of variability/error. The results showed only a weak correlation of fish embryo LC_{50} and rat oral LD_{50} and the inability to effectively predict GHS oral acute toxicity categories. The authors claimed differences of exposure and pharmacokinetics of both systems as the limiting factors.

Objective 1.4. Development of scientifically based waiving arguments to avoid animal testing in acute systemic toxicity studies

The European Commission has recently received proposals by the European Platform for Alternatives to Animal Testing (EPAA) and the Humane Society International (HSI) to modify REACH standard information requirements for acute toxicity. With regard to the acute dermal toxicity test, the EPAA proposal suggested the waiving of a dermal study if an oral LD_{50} is greater than 2000 mg/kg b.w. (i.e. not classified by the oral route). This is supported by publications showing that the overall classification is rarely driven by the dermal classification and substances which are not classified by the oral route are also not classified

for the dermal route (Indans et al, 1998; Thomas & Dewhurst, 2007; Creton et al, 2010; Seidle et al, 2011; Moore et al, 2013).

Other elements of the proposals included recommendations to: a) request acute toxicity testing by routes other than oral only if certain criteria are fulfilled; b) take toxicity and bioavailability into account when deciding whether other routes should be tested; c) test dermal absorption before performing an acute dermal toxicity study; d) establish quantitative criteria to assess the need for acute toxicity testing via the inhalation route; and e) make the acute toxic class method the preferred method for acute toxicity testing via the inhalation route. These proposals have been discussed by the Competent Authorities for REACH and CLP (CARACAL) and at the July 2014 meeting they 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 2000mg/kg body weight would not require dermal data.

A project proposal submitted to the OECD by the USA and Canada aims to develop guidance for waiving or bridging mammalian acute toxicity tests, including acute systemic toxicity testing, for pesticides and biocides. The project was approved by the OECD Working Group of National Coordinators for the Test Guidelines Programme (OECD WNT) and invites the collaboration between several stakeholders.

2.2 Strategic Aim 2: Refinement of animal studies for acute systemic toxicity

The use of death as an endpoint for acute toxicity testing is a matter of concern among many scientists and regulators, not only because of the direct negative impact on animal welfare but also because it has little value in risk assessment and risk management (e.g. derivation of acute DNELs, establishment of acute reference doses) or in deciding treatment of symptoms in human acute toxicity (Chapman et al., 2010; Creton et al., 2010; EPAA, 2012). As encouraged by the Directive 2010/63/EU (EU, 2010) and OECD Guidance Document 19 (OECD, 2000), the substitution of lethality by more humane clinical signs indicative of imminent death would be beneficial on both scientific and animal welfare grounds. At present, only the oral fixed dose procedure (OECD TG420) uses observations of evident clinical signs of toxicity to provide a range estimate of the LD_{50}, thus avoiding death as endpoint.

For the assessment of acute oral and inhalation toxicity there are alternative *in vivo* methods available that represent refinement and/or reduction approaches to testing compared to the deleted oral TG401 (OECD, 1987b) and the standard inhalation TG403 (OECD, 2009a). However, for the assessment of acute dermal toxicity the only guideline currently available is the classic dermal LD_{50} study (TG402; OECD, 1987a) that uses lethality as the primary endpoint and requires an average number of animals between 10 (limit test) and 30.

Objective 2.1. Continue efforts to avoid the use of lethality as endpoint for acute systemic toxicity testing

During an EPAA expert meeting organised in 2012 to review methods used to evaluate acute human toxicity of chemicals and agrochemicals, the need to move away from lethality as the endpoint for acute systemic DNEL derivation was emphasised, not only for obvious ethical reasons but also because of the need to reduce, as far as possible, the level of uncertainty in extrapolating the dose descriptor for DNEL to the human health risk assessment (http://ec.europa.eu/enterprise/epaa/index_en.htm).

An EPAA project on acute systemic toxicity is ongoing and experts are discussing how to address classification and labelling requirements (all routes of exposure) by alternative means. This project is expected to provide useful insight towards the development of waiving arguments (objective 1.4) and the integration of 'evident toxicity' instead of death as an endpoint. In addition, the use of cytotoxicity assays, chemical grouping and read-across, QSARs and data from *in vivo* dose range-finding studies to satisfy regulation is also envisaged.

The UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) is leading activities to support the acceptance at OECD level of the fixed concentration procedure (FCP) for acute inhalation studies (OECD TG433). The proper use of clinical signs instead of lethality for classification and labelling purposes is the basis of the fixed concentration procedure. The NC3Rs project consists of recording clinical signs in acute inhalation studies and to develop and test a new system of scoring clinical signs in acute inhalation studies as a means of robustly identifying evident toxicity (http://www.nc3rs.org.uk/adoption-fixed-concentration-procedure-acute-inhalation-studies).

Objective 2.2. Support the revision of current *in vivo* acute dermal toxicity test

The OECD WNT recently approved a project proposal submitted by the UK to either revise or replace the OECD TG402 (acute dermal toxicity testing) in line with the 3Rs principles. The overall aim is to refine the testing for acute dermal toxicity and reduce the number of animals used. The current guideline requires the use of 5 animals per sex. If the refinements proposed are eventually accepted, OECD Guidance Document 24 (OECD, 2001d) would also need to be updated.

3. Conclusions

This document presents EURL ECVAM's strategy on how to achieve 3Rs impact in the area of acute systemic toxicity assessment and testing. A number of objectives and related activities (not necessarily exhaustive) have been identified to achieve the stated strategic aims and ultimately change the way information requirements are satisfied for the different pieces of EU legislation (i.e. CLP, REACH, Biocidal and Plant Protection Products Regulations). One clear target is the implementation of the REACH Regulation and, in particular, the provision of acute systemic toxicity information requirements for low tonnage chemicals by the 2018 registration deadline.

EURL ECVAM is focusing its in-house activities on evaluating promising components of integrated approaches for testing and assessment, including the better use of alternative (*in vitro* and *in silico*) methods and on exploring the usefulness of existing data from other types of systemic toxicity studies. EURL ECVAM is also exploring the use of *in silico* models in route-to-route and in *in vitro* to *in vivo* extrapolations, and is supporting activities aimed at the refinement of *in vivo* studies through its participation in the EPAA. EURL ECVAM will continue evaluating test method submissions that address this regulatory endpoint in context of the strategy outlined here.

Although EURL ECVAM is committed to play its role, the timely achievement of the objectives and aims presented here will depend on the proactive and coordinated engagement of multiple stakeholders.

4. References

Bessems, J., Coecke, S., Gouliarmou, V., Worth, A., Whelan, M. EURL ECVAM Strategy for Promoting the 3Rs in Human Toxicokinetics (in preparation).

Bulgheroni, A., Kinsner-Ovaskainen, A., Hoffmann, S., Hartung, T., Prieto, P. (2009). Estimation of acute oral toxicity using the No Adverse Effect Level (NOAEL) from the 28-day repeated dose toxicity studies in rats. Regulatory Toxicology and Pharmacology 53: 16-19.

Chapman, K., Creton, S., Kupferschmidt, H., Bond, G.R., Wilks, M.F., Robinson, S. (2010). The value of acute toxicity studies to support the clinical management of overdose and poisoning: A cross-discipline consensus. Regulatory Toxicology and Pharmacology 58: 354–359.

Creton, S., Dewhurst, I.C., Earl, L.K., Gehen, S.C., Guest, R.L., Hotchkiss, J.A., Indans, I., Woolhiser, M.R., Billington, R. (2010). Acute toxicity testing of chemicals—opportunities to avoid redundant testing and use alternative approaches. Critical Reviews in Toxicology 40: 50–83.

Ekwall, B. (1999). Overview of the Final MEIC Results: II. The In Vitro--In Vivo Evaluation, Including the Selection of a Practical Battery of Cell Tests for Prediction of Acute Lethal Blood Concentrations in Humans. Toxicology in Vitro 13(4-5): 665-673.

EPAA (2012). Acute toxicity workshop of the EPAA platform on 3Rs in Regulation: To address the hypothetical situation "How could C&L decisions in the agrochemical and chemical sectors be made if stand-alone acute toxicity testing were prohibited?"

European Commission – EURL ECVAM (2013). EURL ECVAM recommendation on the 3T3 Neutral Red Uptake (3T3 NRU) Cytotoxicity Assay for the Identification of Substances not Requiring Classification for Acute Oral Toxicity. Available at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations

European Union (2006). Regulation (EC) No 1907/2006 of the European Parliament and the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal of the European Union L396: 1-849.

European Union (2008a). Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Official Journal of the European Union L353.

European Union (2008b). Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official Journal of the European Union L142.

European Union (2009a). Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal of the European Union L309: 1-47.

European Union (2009b). Regulation (EC) No 1223/2009 of the European Parliament and the Council of 30 November 2009 on cosmetic products. Official Journal of the European Union L342: 59-209.

European Union (2010). Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European, L276: 33-79.

European Union (2012). Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal of the European Union L 167: 1-116.

European Union (2014). Commission Regulation (EU) No 260/2014 of 24 January 2014 amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official Journal of the European Union L 81, 1-253.

Gajewska, M., Worth, A., Urani, C., Briesen, H., Schramm, K.W. (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. Regul Toxicol Pharmacol. 70(1): 312-324.

Gennari, A., van den Berghe, C., Casati, S., Castell, J., Clemedson, C., Coecke, S., Colombo, A., Curren, R., Dal Negro, G., Goldberg, A., Gosmore, C., Hartung, T., Langezaal, I., Lessigiarska, I., Maas, W., Mangelsdorf, I., Parchment, R., Prieto, P., Sintes, J.R., Ryan, M., Schmuck, G., Stitzel, K., Stokes, W., Vericat, J.A., Gribaldo, L. (2004). Strategies to replace in vivo acute systemic toxicity testing. The report and recommendations of ECVAM Workshop 50. ECVAM Workshop 50. Alternatives to Laboratory Animals 32(4): 437-459.

Halle, W. (2003). The registry of cytotoxicity: toxicity testing in cell cultures to predict acute toxicity (LD50) and to reduce testing in animals. Alternatives to Laboratory Animals 31: 89–198.

Hartung, T. (2008). Food for thought… on animal tests. ALTEX 25: 3-9.

Hartung, T. (2014). Immunotoxicity. *In* In vitro Toxicology systems, Methods in Pahrmacology and Toxicology. (Eds) Bal-Price A. and Jennings, P. Springer Sciences+Busniness Media, New York, pp 241-67.

Hoffmann, S., Kinsner-Ovaskainen, A., Prieto, P., Mangelsdorf, I., Bieler, C., Cole, T. (2010). Acute oral toxicity: variability, reliability, relevance and interspecies comparison of rodent LD50 data from literature surveyed for the ACuteTox project. Regul Toxicol Pharmacol. 58(3): 395-407.

ICH (2000). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guideline on safety pharmacology studies for human pharmaceuticals. Current Step 4 version dated 8 November 2000.

ICH (2009). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals M3(R2). Recommended for Adoption at Step 4 of the ICH Process on June 11, 2009.

Indans, I., Fry, T., Evans, P. (1998). Classification and labelling of new industrial chemicals for acute toxicity, skin and eye irritation. Human and Experimental Toxicology 17: 529.

Kinsner-Ovaskainen, A., Prieto, P., Stanzel, S., Kopp-Schneider, A. (2013). Selection of test methods to be included in a testing strategy to predict acute oral toxicity: an approach based on statistical analysis of data collected in phase 1 of the ACuteTox project. Toxicology In Vitro. 27(4): 1377-94.

Moore, N.P., Andrew, D.J., Bjerke, D.L., Creton, S., Dreher, D., Holmes, T., Prieto, P., Seidle, T., Rowan, T.G. (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). Regulatory Toxicology and Pharmacology 66(1): 30-37.

NIH (2006). Background Review Document (BRD): Validation of Neutral Red Uptake Test Methods NIH /In Vitro Cytotoxicity Test Methods for Estimating Acute Oral Systemic Toxicity. National Institutes of Health Publication No. 07-4518, November 2006. Available at: http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_brd.htm

NIH (2009). The report on the ICCVAM-NICEATM/ECVAM/JaCVAM Scientific Workshop on Acute Chemical Safety Testing: Advancing In Vitro Approaches and Humane Endpoints for Systemic Toxicity Evaluations. National Institutes of Health Available at:

http://ntp.niehs.nih.gov/iccvam/docs/acutetox_docs/workshop-feb09/acutewksprpt.pdf

OECD (1987a). Guideline for Testing of Chemicals 402, Acute Dermal Toxicity, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264070585-en

OECD (1987b). Guideline for Testing of Chemicals 401, Acute Oral Toxicity, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. DOI: 10.1787/9789264040113-en

OECD (2000). Guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation. Series on Testing and Assessment N°19. Organisation for Economic Cooperation and Development Paris, France. Available from: http://www.oecd.org

OECD (2001a). Guideline for Testing of Chemicals, 420, Acute Oral Toxicity – Fixed Dose Method. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264070943-en.

OECD (2001b). Guideline for Testing of Chemicals, 423, Acute Oral Toxicity – Acute Toxic Class Method. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264071001-en.

OECD (2001c). Guideline for Testing of Chemicals, 425, Acute Oral Toxicity – Up-and-Down Procedure. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264071049-en.

OECD (2001d). Guidance document on acute oral toxicity testing. Series on Testing and Assessment N° 24. Organisation for Economic Cooperation and Development Paris, France. Available from: http://www.oecd.org

OECD (2009a). Guideline for Testing of Chemicals, 403, Acute Inhalation Toxicity. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264070608-en

OECD (2009b). Guideline for Testing of Chemicals, 436, Acute Inhalation Toxicity – Acute Toxic Class Method. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264076037-en

OECD (2010). Guidance Document No 129 on using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests. Organisation for Economic Cooperation and Development Paris, France. Available at: http://www.oecd.org

OECD (2013). Guidance document N° 184 on developing and assessing adverse outcome pathways. Organisation for Economic Cooperation and Development Paris, France. Available at: http://www.oecd.org

Pery, A.R.R., Brochot, C., Zeman, F., Mombelli, E., Desmots, S., Pavan, M., Fioravanzo, E., Zaldivar, J-M. (2013). Prediction of dose - hepatotoxic response in humans based on toxicokinetic/toxicodynamic modeling with or without in vivo data: A case study with acetaminophen. Toxicology Letters 220: 26–34.

Prieto, P., Kinsner-Ovaskainen, A., Stanzel, S., Albella, B., Artursson, P., Campillo, N., Cecchelli, R., Cerrato, L., Díaz, L., Di Consiglio, E., Guerra, A., Gombau, L., Herrera, G., Honegger, P., Landry, C., O'Connor, J.E., Páez, J.A., Quintas, G., Svensson, R., Turco, L., Zurich, M.G., Zurbano, M.J., Kopp-Schneider, A. (2013a). The value of selected in vitro and in silico methods to predict acute oral toxicity in a regulatory context: Results from the European Project ACuteTox. Toxicology in Vitro 27(4): 357-376.

Prieto, P., Cole, T., Curren, R., Gibson, R.M., Liebsch, M., Raabe, H., Tuomainen, A.M., Whelan, M., Kinsner-Ovaskainen, A. (2013b). Assessment of the predictive capacity of the 3T3 neutral red uptake cytotoxicity test method to identify substances not classified for acute oral toxicity (LD50 > 2000 mg/kg): results of an ECVAM validation study. Regulatory Toxicology and Pharmacology 65(3): 344-65.

Robinson, S., Delongeas, J.L., Donald, E., Dreher, D., Festag, M., Kervyn, S., Lampo, A., Nahas, K., Nogues, V., Ockert, D., Quinn, K., Old, S., Pickersgill, N., Somers, K., Stark, C., Stei, P., Waterson, L., Chapman, K. (2008). A European pharmaceutical company initiative challenging the regulatory requirement for acute toxicity studies in pharmaceutical drug development. Regulatory Toxicology and Pharmacology 50: 345-352.

Seidle, T., Robinson, S., Holmes, T., Creton, S., Prieto, P., Scheel, J., Chlebus, M. (2010). Cross-sector review of drivers and available 3Rs approaches for acute systemic toxicity testing. Toxicol Sci. 116(2): 382-96.

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

Scholz, S., Ortmann, J., Klüver, N., Léonard, M. (2014). Extensive review of fish embryo acute toxicities for the prediction of GHS acute systemic toxicity categories. Regul Toxicol Pharmacol. 69(3): 572-9.

Schrage, A., Hempel, K., Schulz, M., Kolle, S.N., van Ravenzwaay, B., Landsiedel, R. (2011). Refinement and reduction of acute oral toxicity testing: a critical review of the use of cytotoxicity data. ATLA 39(3): 273-95.

Thomas, H.D., Dewhurst, I.C. (2007). What does a dermal acute toxicity study add to the information on a plant protection pesticide? Toxicology 231: 114–115.

Zbinden, G., and Flury-Roversi, M. (1981). Significance of the LDs0-Test for the Toxicological Evaluation of Chemical Substances. Arch. Toxicol. 47: 77-99.

Zurich, M.G., Stanzel, S., Kopp-Schneider, A., Prieto, P., Honegger, P. (2013). Evaluation of aggregating brain cell cultures for the detection of acute organ-specific toxicity. Toxicology in Vitro 27(4): 1416–1424.

Annex I Regulatory Requirements for Acute Systemic Toxicity

Information on acute systemic toxicity represents a standard requirement within several pieces of chemicals legislation in the EU, as summarised in Table 3. The following sections illustrate the information requirements for acute systemic toxicity within the Regulations considered for the purpose of this report.

1.1 Classification Labelling and Packaging

The Regulation (EC) N° 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP Regulation, EU, 2008a), came into force on the 20th of January 2009 in all EU Member States and aligns previous EU legislation on classification, labelling and packaging of chemicals to the GHS (Globally Harmonised System of Classification and Labelling of Chemicals, UN, 2013). The CLP ensures that the hazards presented by chemicals are clearly communicated to workers and consumers in the EU through classification and labelling of chemicals. This Regulation applies, as a general principle, to all substances and mixtures supplied in the EU except to chemicals that are in the finished state intended for the final user: medicines, medical devices, cosmetics, veterinary medicines, food and feeding stuff such as food additives, food flavouring and feeding stuffs used in animal nutrition.

The term acute toxicity describes the adverse effects observed following the oral or dermal administration of a single dose of a substance or a mixture, or multiple doses given within 24 hours, or an inhalation exposure of 4 hours. Therefore, the hazard class acute toxicity is differentiated into a) **acute oral toxicity**, b) **acute dermal toxicity** and c) **acute inhalation toxicity**.

The CLP Regulation states (annex I, part 3, section 3.1.2.2.1): *the preferred test species for evaluation of acute toxicity by the oral and inhalation routes is the rat, while the rat or rabbit are preferred for evaluation of acute dermal toxicity. When experimental data for acute toxicity are available in several animal species, scientific judgement shall be used in selecting the most appropriate LD_{50} value from among valid, well-performed tests*.

Based on acute toxicity values expressed as (approximate) LD_{50} (oral, dermal) or LC_{50} (inhalation) values or as acute toxicity estimates (ATE), substances can be allocated to one of four toxicity categories according to the numeric criteria shown in Table 1. The acute toxicity label elements for hazard communication are shown in Table 2. Since experimental data may only be available for some of the ingredients of a mixture, specific guidance on classification of mixtures is provided in section 3.1.3 of the CLP Regulation.

It is worth noting that for labelling purposes, the same pictogram (skull and crossbones) and signal word (danger) is used to communicate hazard categories 1 to 3. The hazard statement for categories 1 and 2 is the same (fatal). Prevention, response, storage and

disposal precautionary statements are usually the same for categories 1, 2 and 3. Only categories 1 and 2 for dermal and inhalation routes have more stringent prevention precautionary statements than category 3 (e.g. wear respiratory protection is not foreseen for category 3, see table 2).

Table 1. Acute toxicity hazard categories and acute toxicity estimates (ATE) defining the respective categories depending on the route of exposure (oral, dermal, inhalation)

	ORAL (mg/kg body weight)	**DERMAL** (mg/kg body weight)	**INHALATION**		
			Gases [in parts per million per volume (ppmV)]	**Vapours*** (mg/l)	**Dusts* and Mists*** (mg/l)
Category 1	ATE ≤ 5	ATE ≤ 50	ATE ≤ 100	ATE ≤ 0,5	ATE ≤ 0,05
Category 2	5 < ATE ≤ 50	50 < ATE ≤ 200	100 < ATE ≤ 500	0,5 < ATE ≤ 2,0	0,05 < ATE ≤ 0,5
Category 3	50 < ATE ≤ 300	200 < ATE ≤ 1000	500 < ATE ≤ 2500	2,0 < ATE ≤ 10,0	0,5 < ATE ≤ 1,0
Category 4	300 < ATE ≤ 2000	1000 < ATE ≤ 2000	2500 < ATE ≤ 20000	10,0 < ATE ≤ 20,0	1,0 < ATE ≤ 5,0

*Dust: solid particles of a substance or mixture suspended in a gas (usually air); mist: liquid droplets of a substance or mixture suspended in a gas (usually air); vapour: the gaseous form of a substance or mixture released from its liquid or solid state. Dust is generally formed by mechanical processes. Mist is generally formed by condensation of supersaturated vapours or by physical shearing of liquids. Dusts and mists generally have sizes ranging from less than 1 to about 100 μm (CLP Regulation, section 3.1.2.1)

Table 2. Acute systemic toxicity label elements, i.e. pictograms, signal words, hazard statements and precautionary statements.

Classification	**Category 1**	**Category 2**	**Category 3**	**Category 4**
GHS Pictograms				
Signal Word	Danger	Danger	Danger	Warning
Hazard Statement: **- *Oral*** **- *Dermal*** **- *Inhalation***	H300: Fatal if swallowed H310: Fatal in contact with skin H330: Fatal if inhaled	H300: Fatal if swallowed H310: Fatal in contact with skin H330: Fatal if inhaled	H301: Toxic if swallowed H311: Toxic in contact with skin H331: Toxic if inhaled	H302: Harmful if swallowed H312: Harmful in contact with skin H332: Harmful if inhaled
Precautionary statement - Prevention **- *Oral***	P264, P270			
Precautionary statement - Prevention **- *Dermal***	P262, P264, P270, P280		P280	
Precautionary statement - Prevention **- *Inhalation***	P260, P271, P284		P261, P271	
Precautionary statement - Response **- *Oral***	P301+P310, P321, P330			P301+P312, P330
Precautionary statement - Response **- *Dermal***	P302+P350, P310, P322, P361, P363			P302+P350, P310, P322, P363
Precautionary statement - Response **- *Inhalation***	P304+P340, P310, P320			P304+P340, P312
Precautionary statement - Storage **- *Oral***	P405			

- Dermal ***- Inhalation***	P405 P403+P233, P405	
Precautionary statement - Disposal ***- Oral*** ***- Dermal***	P501 P501	
Precautionary statement - Disposal ***- Inhalation***	P501	

P233: Keep container tightly close
P260: Do not breathe dust/fume/gas/mist/vapours/spray
P262: Do not get in eyes, on skin, or on clothing
P264: Wash….thoroughly after handling
P270: Do not eat, drink or smoke when using this product
P271: Use only outdoors or in a well-ventilated area
P280: Wear protective gloves/protective clothing/eye protection/face protection
P284: Wear respiratory protection
P301 + P310: If swallowed, immediately call a POISON CENTRE or doctor/physician
P302 + P350: If on skin, gently wash with plenty of soap and water
P304 + P340: If inhaled, remove victim to fresh air and keep at rest in a position comfortable for breathing
P312: Call a POISON CENTER or doctor/ physician if you fell unwell
P320: specific treatment is urgent (see…on this label)
P321: specific treatment (see…on this label)
P322: specific measures (see…on this label)
P330: Rinse mouth
P361: Remove/take off immediately all contaminated clothes
P363: Wash contaminated clothes before reuse
P403: Store in a well-ventilated place
P405: Store locked up
P501: Dispose of contents/container to…

1.2 Chemicals

Regulation (EC) N° 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), was adopted in the EU to improve the protection of human health and the environment from the risks that can be posed by chemicals, while enhancing the competitiveness of the EU chemicals industry. It also promotes alternative methods for the hazard assessment of substances in order to reduce animal testing. REACH entered into force on the 1st of June 2007 (EU, 2006). Classification of substances is a mandatory part of the REACH registration process and, therefore, the CLP Regulation and the REACH Regulation are closely interlinked.

The assessment of acute systemic toxicity is among the standard information requirements for substances manufactured or imported into the EU in quantities of 1 tonne or more per year (tpy) affecting, therefore, all chemicals registered under REACH. The standard information requirements for acute toxicity are tonnage triggered and are specified in Annexes VII and VIII as follows:

- **Annex VII** (≥1 tpy): acute toxicity via the oral route of exposure is required. Column 2 of Annex VII details specific rules for adaptation of these information requirements, notably allowing for the waiving of acute oral toxicity testing if the substance is corrosive to the skin or if a study on acute toxicity by the inhalation route is available.
- **Annex VIII** (≥ 10 tpy): as indicated in column 2 of Annex VIII - specific rules for adaptation:

  [par. 8.5]: in addition to the oral route, for substances other than gases, the information mentioned under 8.5.2 to 8.5.3 shall be provided for at least one other route. The choice for the second route will depend on the nature of the substance and the likely route of human exposure. If there is only one route of exposure, information for only that route needs to be provided.

  [par. 8.5.2]: testing by the inhalation route is appropriate if exposure of humans via inhalation is likely, taking into account the vapour pressure of the substance and/or the possibility of exposure to aerosols, particles or droplets of an inhalable size.

  [par. 8.5.3]: Testing by the dermal route is appropriate if:
  (1) inhalation of the substance is unlikely; and
  (2) skin contact in production and/or use is likely; and

  (3) the physicochemical and toxicological properties suggest the potential for a significant rate of absorption through the skin.

Testing in animals does not need to be conducted in case there is available information to classify the substance for acute toxicity or the substance is classified as corrosive for the skin. In case testing is necessary, the *in vivo* methods accepted by regulatory bodies include the acute oral toxicity – fixed dose procedure [OECD TG420 (OECD, 2001a); EU B.1 bis (EU, 2008b)], the acute oral toxicity – acute toxic class method [OECD TG423 (OECD, 2001b); EU B.1tris (EU, 2008b)], the acute oral toxicity – up-and-down procedure [OECD TG425 (OECD,

2001c)]; the acute dermal toxicity [OECD TG402 (OECD, 1987); EU B.3 (EU, 2008b)]; the acute inhalation toxicity [OECD TG403 (OECD, 2009a); EU B.2 (EU, 2014)] , the acute inhalation toxicity - acute toxic class method [OECD TG436 (OECD, 2009b]; EU B.52 (EU, 2014).

Annex I of the REACH Regulation describes how manufacturers and importers of substances have to assess and document that the hazards and potential risks from the substance they manufacture or import are controlled during manufacture and their own use(s) so that others further down the supply chain can adequately control the risks. For hazard assessment, a four step process is described that comprises the evaluation of non-human and human data, the classification and labelling of the substance and the calculation of Derived No-Effect Level (DNEL). The DNEL is defined as the level of exposure which should not be exceeded and is derived from all hazard information available on a substance (REACH Annex I, 1.0.1). Exposure levels of human populations can vary (for examples workers vs. general population) and should be compared to the appropriate DNEL to characterise the risk associated with exposures to a substance, taking into account the likely route(s) of exposure. For systemic acute effects, two DNELs (worker-DNEL acute inhalation and general population-DNEL acute inhalation) are normally required, although occupational inhalation exposure is often the most important one. Rarely, and on a case-by-case basis, the other routes may need to be assessed (potentially constituting three different DNELs). In terms of an acute toxicity DNEL, it has been proposed that the long-term DNEL is normally sufficient to set safe exposure levels for a substance (http://echa.europa.eu/documents/10162/13632/information_requirements_r8_en.pdf).

However, if an acute toxicity hazard has been identified, an acute toxicity DNEL should be established for peak exposures, which may exceed the average daily exposures of long-term or acute DNELs. This is particularly relevant to workers, who may be exposed to a high concentration of a substance, for a short time period, for example when sampling from a vessel.

Chemicals that are imported into or produced in the EU in quantities of 1 tonne or more per year per company have to be registered under REACH with a registration dossier. Several deadlines for registration have been set according to tonnage bands. By 2010 very toxic chemicals and those imported or produced at ≥ 1000 tpy had to be registered and by 31 May 2013 chemicals imported or produced at 100 – 1000 tpy were registered. The final deadline for chemicals imported or produced in the Union at 1 – 100 tpy is the 31st of May 2018. The number of registrations received by 31/10/2014 was 40229 corresponding to 7992 unique substances (ECHA website http://echa.europa.eu/web/guest/information-on-chemicals/registration-statistics).

A precise indication of the likely number of new acute toxicity studies that are anticipated for the 2018 registration is not yet available. Pedersen et al. forecasted in 2003 around 22477 phase-in substances[2] in the tonnage band between 1 and 100 tpy. Comparing the estimates

[2] Phase-in substances are substances that were already manufactured or placed on the market before REACH's entry into force

made for the 2010 and 2013 registration deadlines (2704 and 2461 substances, respectively) and the final outcome of both registrations (3400 and 2998 substances registered, respectively), and assuming that the same trend is maintain (~1.2 increase rate), one could expect around 26972 substances registered for the 2018 deadline. Moreover, according to the report prepared by Van der Jagt et al (2004) using a standard scenario that assumed possible use of QSARs, grouping, read across and options for waiving, it was forecasted that less than 5% of the phase-in substances would require testing on acute systemic toxicity (similar estimation for each route of exposure). The first ECHA report on the status of non-animal methods and alternative testing strategies used to generate information for registration purposes (Article 117(3) of REACH Regulation) showed that a substantial number of new *in vivo* studies were submitted to fill the data gaps for Annex VII and VIII endpoints that do not require testing proposals. In total, 1789 substances were considered by ECHA in the analysis carried out for this first report. Table 2 of the report shows that for acute toxicity, 486 new studies were identified: 211 by oral route, 114 by inhalation route and 161 by the dermal route, which would result in 13%, 6% and 9% of substances requiring new acute toxicity tests by the oral, inhalation and dermal route, respectively (ECHA, 2011). On the basis of all these assumptions, one could roughly estimate that for the next registration deadline no more than about 3500, 1600 and 2400 substances will require new information on acute oral, inhalation and dermal systemic toxicity, respectively. ECHA has published recently the second report (ECHA, 2014) that covered a total number of 3662 substances. Overall the number of new experimental studies has increased twice compared to the data published in 2011. In line with previous report most the of all new studies were submitted to fill in the data gaps for the Annex VII and VIII endpoints for which testing proposals were not required (among them acute systemic toxicity). From the new 1153 new acute toxicity studies identified with the date of 2009 or later, 464 were performed via the oral route, 468 via the dermal route and 221 via the inhalation route.

1.3 Biocides

The Biocidal Products Regulation (BPR, Regulation (EU) 528/2012) was adopted on the 22nd of May 2012 and came into force on the 1st of September 2013 (EU, 2012). It governs the toxicological testing, placing on the market, and use of biocidal products. Biocidal products contain active substances and are used to protect humans, animals, materials or articles from harmful organisms such as pests or bacteria. First and foremost the BPR aims to offer a high level of protection to humans and the environment. It also aims to harmonise the EU market as well as promote the reduction of animal testing by encouraging data sharing and the use of alternative testing methods.

The information requirements for active substances and biocidal products are set out in Annexes II and III of the BPR, respectively. A detailed guidance on the application of these annexes and the preparation of the dossiers is available from the ECHA website (http://echa.europa.eu/documents/10162/15623299/biocides_guidance_information_requirements_en.pdf). A stepwise approach for fulfilling information requirements is described in

this guidance document, where the first two steps comprise the gathering and analysis of all available information, such as physicochemical properties and QSAR predictions, on the active substance. Next, if necessary, guided by the information from the first two steps, new data is generated in the third step and in the final step this data is analysed (ECHA, 2013).

Data requirements for **active substances** are reported as follows (Annex II, par. 8.7):

- *In addition to the oral route of administration (8.7.1), for substances other than gases, information mentioned under 8.7.2 to 8.7.3 shall be provided for at least one other route of administration.*
- *The choice for the second route will depend on the nature of the substance and the likely route of human exposure. Gases and volatile liquids should be administered by the inhalation route.*
- *If the only route of exposure is the oral route, then information for only that route need be provided. If either the dermal or inhalation route is the only route of exposure to humans then an oral test may be considered. Before a new dermal acute toxicity study is carried out, an in vitro dermal penetration study (OECD 428) should be conducted to assess the likely magnitude and rate of dermal bioavailability.*
- *There may be exceptional circumstances where all routes of administration are deemed necessary.*

If a substance is classified as being corrosive to skin it does not need to be tested for acute toxicity (column 3 – specific rules for adaptation).

By oral route (par. 8.7.1):

- *The Acute Toxic Class Method is the preferred method for the determination of this endpoint.*

Testing by the oral route is not necessary if the substance is a gas or a highly volatile substance (column 3 – specific rules for adaptation).

With regard to the decision on the protocol to follow for this endpoint, the ECHA guidance document indicates that animal welfare issues should be taken into account and that the fixed dose procedure (i.e. OECD TG 420) should be considered as the first choice for testing (note that the information on the guidance document does not constitute legal advice).

Inhalation route (par. 8.7.2.): Testing by the inhalation route is appropriate if human exposure is likely via inhalation, taken into account if:

- *the vapour pressure of the substance (a volatile substance has vapour pressure > 1 x 10^{-2} Pa at 20 °C) and/or*
- *the active substance is a powder containing a significant proportion (e.g. 1 % on a weight basis) of particles with particle size MMAD (Mass Median Aerodynamic Diameter) < 50 micrometers or*

- *the active substance is included in products that are powders or are applied in a manner that generates exposure to aerosols, particles or droplets of an inhalable size (MMAD <50 micrometers)*
- *The Acute Toxic Class Method is the preferred method for the determination of this endpoint.*

The ECHA guidance document states that even if there is no information on particle/droplet size, an acute inhalation study should be performed where there is potential for exposure via inhalation from the use of biocidal products containing the active substance. With regard to the exposure conditions, the guidance specifies that unless whole body exposure is justified, only the head/nose of the animal should be exposed. In case the limit concentration of the test guideline or a maximum attainable concentration of the substance does not produce compound-related mortalities a full, three dose study may not be necessary (section 8.7.2 ECHA Guidance on information requirements; ECHA, 2013).

Dermal route (par. 8.7.3): Testing by the dermal route is necessary only if:

- *inhalation of the substance is unlikely, or*
- *skin contact in production and/or use is likely, and either*
- *the physicochemical and toxicological properties suggest potential for a significant rate of absorption through the skin, or*
- *the results of an in vitro dermal penetration study (OECD 428) demonstrate high dermal absorption and bioavailability*

The ECHA guidance document specifies that new OECD validated tests for acute dermal toxicity should be taken into account once available and similarly validated non-animal methods should be consulted. For substances with low acute dermal toxicity a limit test with 2000 mg/kg body weight may be sufficient (section 8.7.3 ECHA Guidance on information requirements; ECHA, 2013).

Information requirements for **biocidal products** are reported as follows (Annex III):

- *(par. 8.5): Classification using the tiered approach to classification of mixtures for acute toxicity in Regulation (EC) No 1272/2008 is the default approach.*
- *(par. 8.5; column 3 – specific rules for adaptation) Testing on the biocidal product/mixture does not need to be conducted if there are valid data available on each of the components in the mixture to allow classification according to the rules laid down in Directive 1999/45/EC and Regulation (EC) No 1272/2008 (CLP), and synergistic effects between any of the components are not expected.*
- *(par. 8.5.4): For biocidal products that are intended to be authorised for use with other biocidal products, the risks to human health, animal health and the environment arising from the use of these product combinations shall be assessed. As an alternative to acute toxicity studies, calculations can be used. In some cases, for example where there are no*

valid data available of the kind set out in column 3, this may require a limited number of acute toxicity studies to be carried out using combinations of the products.

- *(par. 8.5.4; column 3 – specific rules for adaptation): Testing on the mixture of products does not need to be conducted if there are valid data available on each of the components in the mixture to allow classification of the mixture according to the rules laid down in Directive 1999/45/EC and Regulation (EC) No 1272/2008 (CLP), and synergistic effects between any of the components are not expected.*

1.4 Plant Protection Products

The Regulation (EC) N° 1107/2009 concerns the placing of plant protection products (PPPs) on the market (EU, 2009a). It came into force on the 21st of October 2009. PPPs describe a range of products such as insecticides, fungicides and plant growth regulators that are applied to plants and crops before and/or after their harvest in order to protect and preserve them. Maximum pesticide residues levels that may be present in food are regulated by Regulation (EC) No 396/2005[3] and fall outside the scope of this document. PPPs contain active substances that have to be tested in terms of their safety for human health, animal health and the environment. Active substances that are deemed to be safe are placed on an EU list and Member states may authorise only PPPs that contain active substances from this list.

The data requirements for the **active substances** of PPPs are set out in Commission Regulation (EU) N° 283/2013 that came into effect on the 1st of March 2013 (EU, 2013a). In terms of acute toxicity testing, section 5.2 states the following:

The studies, data and information to be provided and evaluated shall be sufficient to permit the identification of effects following a single exposure to the active substance, and in particular to establish, or indicate:

a. *the toxicity of the active substance;*
b. *the time course and characteristics of the effect with full details of behavioural changes and possible gross pathological findings at post-mortem;*
c. *the possible need to consider establishing acute reference doses (such as the acute reference dose [ARfD], the acute acceptable operator exposure level [AOEL]);*
d. *where possible the mode of toxic action; and*
e. *the relative hazard associated with the different routes of exposure*.

The ARfD of a pesticide describes the amount that can be ingested by humans in a period of 24 hours or less without any appreciable health risk (Yoshida et al., 2013). Ideally, an ARfD would be set based on an acute toxicity study, however these studies only provide limited

[3] REGULATION (EC) NO 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC

information. In order to avoid carrying out another toxicity study, the NOAEL value for a relevant endpoint of a repeated dose toxicity study is mostly used to set an ARfD. For example, reproductive and developmental toxicity, acute neurotoxicity and haematotoxicity studies have been shown to provide relevant information for setting ARfD values (Yoshida et al., 2013, Solecki et al., 2005). An appropriate safety factor (ranging from 10 – 100) is then applied to the NOAEL value to set the ARfD (Solecki et al., 2005).

The information should allow classification of the substance in accordance with the CLP Regulation (EC N° 1272/2008). The information generated is of particular value in assessing hazards likely to arise in accident situations. All available data that is relevant for the assessment of the toxicological profile of the active substance such as physicochemical properties, biological data and structure-activity relationships of chemical analogues shall be provided. Only validated methods that are specific to the endpoint under investigation should be used in the toxicity studies.

The use of non-animal test methods and other risk assessment strategies is promoted in order to keep the number of animals used for testing to a minimum and to use animal testing as a last resort.

Circumstances in which oral route is required (par. 5.2.1):

The acute oral toxicity of the active substance shall always be reported.

Circumstances in which dermal route is required (par. 5.2.2):

Acute dermal toxicity studies need to be reported, unless waiving is scientifically justified, for example where oral LD_{50} is greater than 2000 mg/kg. Both local and systemic effects need to be investigated. Findings of severe skin irritation or corrosion in the dermal study may be used instead of performing a specific irritation study.

Circumstances in which inhalation route is required (par. 5.2.3):

Acute inhalation toxicity studies are required where any of the following apply:

a) *the active substance has a vapour pressure > 1 × 10 –2 Pa at 20 °C;*
b) *the active substance is a powder containing a significant proportion of particles of a diameter < 50 µm (> 1 % on weight basis);*
c) *the active substance is included in products that are powders or are applied by spraying.*

The head/nose only exposure shall be used, unless whole body exposure can be justified.

The data requirements for **plant protection products** are set out in Commission Regulation (EU) N° 284/2013 that came into effect on the 1st of March 2013 (EU, 2013b). Information on acute toxicity shall be provided. The Regulation states that *the relevant calculation methods used for the classification of mixtures as laid down in Regulation (EC) N° 1272/2008 shall, where appropriate, be applied in the hazard assessment of the plant protection product.*

1.5 Cosmetics

Regulation (EC) N° 1223/2009 on cosmetic products came into force in December 2009 and is fully applicable since the 11th of July 2013 (EU, 2009b). According to Article 1, the Regulation *establishes rules to be complied with by any cosmetic product made available on the market, in order to ensure the functioning of the internal market and a high level of protection of human health.* Since coming into force, the Regulation prohibits (article 18) the placing on the market of:

- *cosmetic products where the final formulation has been the subject of animal testing*;
- *cosmetic products containing ingredients or combinations of ingredients which have been the subject of animal testing*.

When a cosmetic product is placed on the market a product information file should be available and shall contain a cosmetic product safety report, which in turn shall contain as a minimum the cosmetic product safety information and safety assessment (Annex I of Regulation (EC) N° 1223/2009). With regard to safety information, Annex I of the legal text states that the report shall contain *without prejudice to Article 18* (animal testing)*, the toxicological profile of substance contained in the cosmetic product for all relevant toxicological endpoints. A particular focus on local toxicity evaluation (skin and eye irritation), skin sensitisation, and in the case of UV absorption photo-induced toxicity shall be made*. It also states that *particular consideration shall be given to any possible impacts on the toxicological profile due to particle sizes, including nanomaterials, impurities of the substances and raw material used, and interaction of substances*.

In the EU, two channels function with respect to the safety evaluation of cosmetic substances (SCCS, 2012). It is primarily the substances listed in Annexes II, III, IV, V and VI of the cosmetics Regulation that fall under the responsibility of the Scientific Committee on Consumer Safety (SCCS). All ingredients of cosmetic products other than the substances present in the Annexes, is the responsibility of the "responsible person", as defined by the Regulation through the safety assessor. In general, the safety evaluation of cosmetic substances by the SCCS is based on the principles and practice of the risk assessment process (WHO 2001; European Commission 2000) usually applied for ingredients in medicinal products, plant protection products, food additives.

Acute toxicity is part of the minimal base set requirements that a dossier of a cosmetic substance should include if submitted for evaluation to the SCCS (SCCS, 2012).

For all other potential ingredients of cosmetic products outside the Annexes some general toxicological requirements apply. Several cosmetic substances belong to the category of chemical substances EU produced/imported at levels between 1 and 10 tonnes per year. Therefore a sound safety evaluation should at least include REACH data requirements under ANNEX VII (e.g. acute toxicity via the oral route).

Cosmetic products containing substances that have been subject to acute toxicity testing after 11 March 2009 to meet the requirements of the Cosmetic Regulation, are not allowed

on the EU market (SCCS, 2012). This also includes information available from experiments carried out to meet the requirements of the Cosmetic Regulation that have been carried out before the Regulation became applicable. The majority of ingredients used in cosmetic products are also used in many other consumer and industrial products. In this regard, acute toxicity data of cosmetic substances are usually available as a result of compliance with the provisions of other EU Regulations, e.g. the CLP Regulation (EU, 2008a) and REACH requirements (EU, 2006). Although the data available in these cases may have been generated in animals, they should not trigger marketing ban, since they have not been generated to meet the requirements of the Cosmetic Regulation.

According to the Impact Assessment on the Animal Testing Provisions in Regulation (EC) 1223/2009 on Cosmetics, *acute toxicity plays in practice a limited role for the cosmetics industry. Ingredients used in this sector essentially do not raise the risk of acute toxicity and sufficient information is often available from repeated dose studies.* (http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/animal_testing/ia_at_2013_en.pdf).

1.6 Pharmaceuticals – ICH Guideline

The ICH guidance on non-clinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals [ICH M3(R2)], came into effect in December 2009 (ICH, 2009). This guideline aims to harmonise the testing requirements between Europe, the US and Japan, further implement the principles of the 3Rs and promote the safe and ethical development of new pharmaceutical agents. The testing strategies for the pharmaceutical agents under study should be guided by scientific and ethical principles and may need to be adapted for the specific type of agent under study. Non-clinical safety studies should be adequate to identify the toxic potential of a substance and to aid the identification of an initial safe starting dose for human trials.

Information on acute toxicity of pharmaceutical compounds used to be obtained from single dose toxicity studies in two mammalian species using both the clinical and a parenteral route of exposure. However, it has been realised that such information can be obtained from appropriately conducted dose-escalation studies or short-duration dose-ranging studies that define a maximum tolerated dose in the general toxicity test species (Robinson et al., 2008). If this acute toxicity information is available, no additional single dose acute toxicity studies are recommended thus reducing the overall number of studies and animals used. For example, appropriately conducted dose escalation studies or short duration dose-ranging studies that define a maximum tolerated dose in the general toxicity test species can provide information on acute toxicity. In all cases a dose of 1000 mg/kg/day in rodent and non-rodent species is considered an appropriate limit dose. Studies can be limited to the clinically relevant route only and data can be obtained from non-GLP studies if clinical administration is supported by GLP repeated dose toxicity studies. Lethality should not be an intended endpoint in studies assessing acute toxicity. In some cases, such as exploratory clinical studies, the acute toxicity or single dose studies can be the primary support for single dose

studies in humans, in which case the high dose selection can be different from that described above but should be adequate to support the intended clinical route and dose.

With regard to pharmaceuticals for veterinary use, Directive 2009/9/EC lays down the data requirements for marketing authorisation applications for veterinary medicinal products. Part 3 of Directive 2009/9/EC, on safety and residues tests, indicates that single dose toxicity studies may be used to predict possible effects of acute overdosage in the target species, possible effects of accidental administration to humans and to provide information on the doses to be used in repeat dose studies. *Single-dose toxicity studies should reveal the acute toxic effects of the substance and the time course for their onset and remission. The studies to be carried out shall be selected with a view to providing information on user safety, e.g. if substantial exposure by inhalation or dermal contact of the user of the veterinary medicinal product is anticipated, those routes of exposure shall be studied*. Only minimal additional guidance is available.

The Committee for Medicinal Products for Veterinary Use (CVMP) guideline on user safety for pharmaceutical veterinary medicinal products highlights that, in relation to user safety, toxicity data from published literature or from toxicity studies may be used (EMA/CVMP/543/03-Rev.1).

In addition, the CVMP guideline on safety and residue data requirements for veterinary medicinal products intended for minor uses or minor species (MUMS) compares the data requirements for standard applications and those for MUMS applications. In relation to standard applications the document indicates that data from two mammalian species would normally be expected but that one species may be the target species, and that data from two routes of administration would normally be expected. The document also indicates that "to reduce animal numbers, alternative validated protocols and internationally recognised protocols will be accepted" (EMEA/CVMP/SWP/66781/2005)

Table 3. Overview of the EU legislations considered in the context of this report and their information requirements for acute systemic toxicity.

EU Regulation	Application	Information requirements	Method of choice
CLP Regulation (EC) N°1272/2008 on the classification, labelling and packaging of substances and mixtures. CLP aligns previous EU legislation to the GHS.	All substances and mixtures supplied in the EU except to chemicals in the finished state intended for the final users. All substances subject to REACH are also subject to classification, even those not placed on the market if they are subject to registration or notification.	Hazard assessment and appropriate labelling. Hazard class acute toxicity is differentiated into acute oral, dermal and inhalation toxicity. For each route of exposure it allows classification into four hazard categories based on acute toxicity values or acute toxicity estimates.	***Oral route:*** Fixed dose procedure (OECD TG 420; EU B.1 bis); Acute toxic class method (OECD TG 423; EU B.1tris); Up-and-down procedure (OECD TG 425); ***Dermal route:*** the acute dermal toxicity (OECD TG 402; EU B.3); ***Inhalation route:*** the acute inhalation toxicity (OECD TG 403; EU B.2); Acute Toxic class method (OECD TG 436)
REACH Regulation (EC) N° 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals.	Acute systemic toxicity is mandatory for substances manufactured or imported in the EU in quantities of 1 tonne or more (i.e. all chemicals registered under REACH). Standard information requirements are tonnage triggered: Testing via the oral route is requested for substances in the tonnage ban 1-10 tpy.	Hazard assessment A DNEL for acute toxicity should be derived if an acute toxicity hazard (leading to classification and labelling) has been identified and there is a potential for high peak exposures. High peak exposures are usually assessed for the inhalation route only.	***Oral route:*** Fixed dose procedure (OECD TG 420; EU B.1 bis); Acute toxic class method (OECD TG 423; EU B.1tris); Up-and-down procedure (OECD TG 425); ***Dermal route:*** the acute dermal toxicity (OECD TG 402; EU B.3); ***Inhalation route:*** the acute inhalation toxicity (OECD TG 403; EU B.2); Acute Toxic class method (OECD TG 436)

	For substances imported or produced in quantities above 10 tpy, information for at least one other route in addition to the oral route is requested.		
Biocides Regulation (EU) 528/2012 governs the toxicological testing, placing on the market, and use of biocidal products.	All biocidal products and active substances marketed in EU independent of the tonnage level.	Hazard assessment For substances other than gases, information on acute oral toxicity and at least one additional route of exposure is required for both active substances contained in biocidal products and the final product.	***Oral route:*** the acute toxic class method (OECD TG 423; EU B.1tris) is the preferred method. ***Dermal route:*** the acute dermal toxicity (OECD TG 402; EU B.3). ***Inhalation route:*** the acute toxic class method (OECD TG 436) is the preferred method. For biocidal products, the tiered approach to classification of mixtures for acute toxicity in CLP Regulation is the default approach. In the case of product combinations and if data are available in each of the components, calculations are possible.
Plant Protection product Regulation (EC) N° 1107/2009 concerning the placing of plant protection products in the market Regulations (EU) 283/2013 and (EU) No	All plant protection products and active substances marketed in EU independent of tonnage level.	Hazard assessment Information requirement for acute toxicity is mandatory for both active substances contained in PPPs and the final product. For final product relevant calculation methods used for the classification of mixtures as laid down in REACH can be	***Oral route:*** Fixed dose procedure (OECD TG 420; EU B.1 bis); Acute toxic class method (OECD TG 423; EU B.1tris); Up-and-down procedure (OECD TG 425); ***Dermal route:*** the acute dermal toxicity (OECD TG 402; EU B.3); ***Inhalation route:*** the acute

284/2013 set out the data requirements for active substances and plant protection products (PPPs), respectively, in accordance with Regulation (EC) N° 1107/2009.		applied if appropriated. For active substances acute oral toxicity is always required; dermal toxicity unless waiving is scientifically justified and inhalation toxicity only if some quantitative criteria are met.	inhalation toxicity (OECD TG 403; EU B.2); Acute Toxic class method (OECD TG 436)
Cosmetics Regulation (EC) N° 1223/2009	All cosmetic ingredients independent of tonnage level. Acute toxicity is part of the minimal base set requirements.	According to the Impact Assessment on the Animal Testing Provisions in Regulation (EC) 1223/2009 on Cosmetics, *acute toxicity plays in practice a limited role for the cosmetics industry. Ingredients used in this sector essentially do not raise the risk of acute toxicity and sufficient information is often available from repeated dose studies.*	Alternative non-animal methods validated and adopted at Community level.

References to Annex I

ECHA, European Chemicals Agency (2011). The Use of Alternatives to Testing on Animals for the REACH Regulation. ECHA-11-R-004.2-EN

ECHA, European Chemicals Agency (2014). The Use of Alternatives to Testing on Animals for the REACH Regulation. ECHA-14-A-07-EN.

ECHA, European Chemicals Agency (2013). Guidance on information requirements. Guidance on regulation (EU) no 528/2012 concerning the making available on the market and use of biocidal products (BPR) Version 1.0 July 2013. ECHA-13-G-04-EN.

European Commission (2009). Commission Directive 2009/9/EC of 10 February 2009 amending Directive 2001/82/EC of the European Parliament and of the Council on the Community code relating to medicinal products for veterinary use. Official Journal of the European Union L44, 10-61.

European Medicine Agency (2007). Guideline on safety and residue data requirements for veterinary medicinal products intended for minor uses or minor species. Committee for medicinal products for veterinary use (CVMP). EMEA/CVMP/SWP/66781/2005.

European Medicine Agency (2010). Guideline on user safety for pharmaceutical veterinary medicinal products. Committee for medicinal products for veterinary use (CVMP). EMA/CVMP/543/03-Rev.1

European Union (1999). Directive 1999/45/EC of the European Parliament and of the Council of 31 May 1999 concerning the approximation of the laws, regulations and administrative provisions of the Member States relating to the classification, packaging and labelling of dangerous preparations. Official Journal of the European Union L0045: 1-77.

European Union (2000). First Report on the Harmonisation of Risk Assessment Procedures, Part 1. The Report of the Scientific Steering Committee's Working Group on Harmonisation of Risk Assessment Procedures in the Scientific Committees advising the European Commission in the area of human and environmental health 26-27 October 2000 (published on the Internet 20.12.2000).

European Union (2006). Regulation (EC) No 1907/2006 of the European Parliament and the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal of the European Union L396, 1-849.

European Union (2008a). Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Official Journal of the European Union L353.

European Union (2008b). Council regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to regulation (EC) no. 1907/2006 of the European Parliament and of the Council on the registration, evaluation, authorisation and restriction of chemicals (REACH). Official Journal of the European Union L142:1–739.

European Union (2009a). Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal of the European Union L309, 1-47.EC N° 1107/2009

European Union (2009b). Regulation (EC) No 1223/2009 of the European Parliament and the Council of 30 November 2009 on cosmetic products. Official Journal of the European Union L342, 59-209.

European Union (2012). Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal of the European Union L 167, 1-116.

European Union (2013a). Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. Official Journal of the European Union L93, 1-84.

European Union (2013b). Regulation (EU) No 284/2013 of 1 March 2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. Official Journal of the European Union L 93, 85-152.

European Union (2014). Commission Regulation (EU) No 260/2014 of 24 January 2014 amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official Journal of the European Union L 81, 1-253.

ICH, 2009. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals M3(R2). Recommended for Adoption at Step 4 of the ICH Process on June 11, 2009.

OECD (1987). Guideline for Testing of Chemicals 402, Acute Dermal Toxicity. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264070585-en.

OECD (2001a). Guideline for Testing of Chemicals, 420, Acute Oral Toxicity – Fixed Dose Method. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264070943-en.

OECD (2001b). Guideline for Testing of Chemicals, 423, Acute Oral Toxicity – Acute Toxic Class Method. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264071001-en.

OECD (2001c). Guideline for Testing of Chemicals, 425, Acute Oral Toxicity – Up-and-Down Procedure. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264071049-en.

OECD (2004), Guideline for Testing of Chemicals 428, Skin Absorption: In Vitro Method, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264071087-en

OECD (2009a). Guideline for Testing of Chemicals, 403, Acute Inhalation Toxicity. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264070608-en

OECD (2009b). Guideline for Testing of Chemicals, 436, Acute Inhalation Toxicity – Acute Toxic Class Method. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264076037-en

Pedersen, F., de Bruijn, J., Munn, S., van Leeuwen, K. (2003). Assessment of additional testing needs under REACH - effects of (Q)SARs risk based testing and voluntary industry initiatives. Report EUR 20863 EN.

Robinson, S., Delongeas, J.L., Donald, E., Dreher, D., Festag, M., Kervyn, S., Lampo, A., Nahas, K., Nogues, V., Ockert, D., Quinn, K., Old, S., Pickersgill, N., Somers, K., Stark, C., Stei, P., Waterson, L.,

Chapman, K. (2008). A European pharmaceutical company initiative challenging the regulatory requirement for acute toxicity studies in pharmaceutical drug development. Regulatory Toxicology and Pharmacology 50: 345-352.

SCCS, Scientific Committee on Consumer Safety (2012). The SCCS's notes of guidance for the testing of cosmetic substances and their safety evaluation 8th revision. Available from: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_s_006.pdf

Solecki, R., Davies, L., Dellarco, V., Dewhurst, I., Raaij, M.v., Tritscher A. (2005). Guidance on setting of acute reference dose (ARfD) for pesticides. Food Chem Toxicol. 2005 43(11): 1569-93.

United Nations, 2013. Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Filth revised edition. Part 3: "Health hazards". Available online: http://www.unece.org/fileadmin/DAM/trans/danger/publi/ghs/ghs_rev05/English/ST-SG-AC10-30-Rev5e.pdf

Van der Jagt, K., Munn, S., Tørsløv, J., de Bruijn, J. (2004). Alternative approaches can reduce the use of test animals under REACH. Addendum to the report: Assessment of additional testing needs under REACH Effects of (Q)SARS, risk based testing and voluntary industry initiatives. Report EUR 21405 EN. Available from: http://publications.jrc.ec.europa.eu/repository/handle/111111111/8790

World Health Organisation (2001). Approaches to Integrated Risk Assessment, Doc. WHO/IPCS/IRA/01/12 of December 2001. Available from: http://www.who.int/ipcs/publications/new_issues/ira/en/

Yoshida, M., Suzuki, D., Matsumoto, K., Shirota, M., Inoue, K., Takahashi, M., Morita, T., Ono, A. (2013). Simulation of acute reference dose (ARfD) settings for pesticides in Japan. J Toxicol Sci. 38(2): 205-14.

European Commission
EUR 26704 EN – Joint Research Centre – Institute for Health and Consumer Protection

Title: **EURL ECVAM strategy to replace, reduce and refine the use of animals in the assessment of acute mammalian systemic toxicity**

Author(s): Pilar Prieto, Julien Burton, Rabea Graepel, Anna Price, Maurice Whelan, Andrew Worth

Luxembourg: Publications Office of the European Union

2014 – 42 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1831-9424 (online)

ISBN 978-92-79-38867-5 (PDF)

doi:10.2788/86684

LB-NA-26704-EN-N

JRC Mission

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new methods, tools and standards, and sharing its know-how with the Member States, the scientific community and international partners.

Serving society
Stimulating innovation
Supporting legislation

doi:10.2788/86684

ISBN 978-92-79-38867-5

Publications Office