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Guidance on Information Requirements and Chemical Safety Assessment

Chapter R.7a: Endpoint specific guidance

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NOTE

Please note that the present document is a proposed amendment to specific extracts **only** of the *Guidance on IR&CSA, Chapter R.7a*. This document was prepared by the ECHA Secretariat for the purpose of this consultation and includes only the parts open for the current consultation, i.e. section R.7.4 only.

The full document (version before proposed amendments) is available on the ECHA website at http://echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf (version 4.0 published in July 2015).

The numbering and headings of the sub-sections that are displayed in the document for consultation correspond to those used in the currently published guidance document; this will enable the comparison of the draft revised sub-sections with the current text if necessary.

After conclusion of the consultation and before final publication the updated sub-sections will be implemented in the full document.

Version	Changes	Date
Draft Version 5.0	Full revision addressing the content of Section R.7.4 related to Acute toxicity. The update includes the following: • Addition of a new Appendix R.7.4-1 "Weight-of-Evidence based adaptation of the standard information requirement on acute oral toxicity study"; • Update of the information on non-testing methods and detailed description of (Q)SARs for Acute toxicity prediction moved to a new Appendix R.7.4-2; • Update of the information on *in vitro* test methods; • Update of Figure R.7.4-1 on the testing and assessment strategy for acute toxicity and Figure R.7.4-2 on the selection of additional routes of exposure; • Re-numbering of some sub-sections.	XXX 201X

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R.7.4 Acute toxicity

R.7.4.1 Introduction

Assessment of the acute toxic potential of a substance is necessary to determine the adverse health effects that might occur following accidental or deliberate short-term exposure. The nature and severity of the acute toxic effects are dependent upon various factors, such as the mechanism of toxicity and bioavailability of the substance, the route and duration of exposure and the total amount of substance to which the person or animal is exposed.

R.7.4.1.1 Definition of acute toxicity

The term *acute toxicity* is used to describe the adverse effects, which may result from a single exposure (i.e. a single exposure or multiple exposures within 24 hours) to a substance. In the context of this guidance, exposure relates to the oral, dermal or inhalation routes. The adverse effects can be seen as clinical signs of toxicity (for animals, refer to OECD Guidance Document 19 (OECD, 2000)), abnormal body weight changes, and/or pathological changes in organs and tissues, which in some cases may result in death. In addition to acute systemic effects, some substances may have the potential to cause local irritation or corrosion of the gastro-intestinal tract, skin or respiratory tract following a single exposure. Acute irritant or corrosive effects due to the direct action of the substance on the exposed tissue are not specifically covered by this document, although their occurrence may contribute to the acute toxicity of the substance and must be reported. The endpoints of skin corrosion/irritation, serious eye damage/eye irritation and respiratory tract corrosion/irritation are addressed in Section R.7.2 of this Guidance.

At the cellular level acute toxicity can be related to three main types of toxic effect, (i) general basal cytotoxicity (ii) selective cytotoxicity and (iii) cell-specific function toxicity. Acute toxicity may also result from substances interfering with extracellular processes (Seibert, 1996). Toxicity to the whole organism also depends on the degree of dependence of the whole organism on the specific function affected.

R.7.4.1.2 Objective of the guidance on acute toxicity

A substance may induce systemic and/or local effects. This document is concerned with assessment of systemic effects following acute exposure.

Generally the objectives of this Gudiance are to establish:

- whether a single exposure (or multiple exposures within 24 hours) to the substance of interest (when admistered up to the limit dose of 2000 mg/kg bw) could be associated with adverse effects on human health; and/or
- what types of toxic effects are induced, their time of onset, duration and severity (all to be related to dose); and/or
- the dose-response relationships to determine the LD_{50}, the LC_{50}, the discriminating dose, or the acute toxicity category; and/or
- when possible, the slope of the dose-response curve; and/or
- when possible, whether there are marked sex differences in response to the substance; and

- the classification and labelling of the substance for acute toxicity.

The indices of LD_{50} and LC_{50} are derived values relating to the dose that is expected to cause death in 50% of treated animals in a given period; these values do not provide information on all aspects of acute toxicity. Other parameters and observations and their type of dose-response may yield valuable information. The potential to avoid acute toxicity testing should be carefully exploited by application of read-across or other non-testing means. Furthermore, there is an overriding obligation to minimise the use of animals in any assessment of acute toxicity. To this end, Appendix R.7.4-1 on a *Weight-of-Evidence* (*WoE*) adaptation of the standard information requirement for an acute oral toxicity study should be considered. That WoE adaptation may be applied in specific cases, in order to avoid *in vivo* acute toxicity test.

For risk assessment, further considerations on the nature and reversibility of the toxic effects are necessary.

R.7.4.2 Information requirements for acute toxicity

The standard information requirements for acute toxicity under the REACH Regulation are as follows:

Annex VII (≥1 t/y): acute toxicity via the oral route of exposure is required (Section 8.5.1);

Column 2 of Section 8.5 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.

Annexes VIII -X (≥ 10 t/y): acute toxicity *via* the oral and dermal or inhalation route of exposure (Sections 8.5.2 and 8.5.3).

Column 2 of Annex VIII details specific rules for adaptation, notably requiring information on at least one other route of exposure depending on the nature of the substance and the likely route of human exposure (for details see Annex VIII, Section 8.5); as for Annex VII, allowance is made for the waiving of acute oral toxicity testing if the substance is corrosive to the skin.

The registrant has an obligation to perform animal tests only as a last resort, pursuant to Articles 13(1) and 25 of the REACH Regulation. This Guidance, and Appendix R.7.4-1 in particular, can help the registrant determine whether any non-animal or non-testing approach could be used instead of *in vivo* testing in order to meet the relevant information requirements.

R.7.4.3 Information sources on acute toxicity

Information on acute toxicity, as detailed below, can be obtained from a variety of sources including unpublished studies, data bases and publications such as books, scientific journals, criteria documents, monographs and other publications (see Chapter R.3 of the *Guidance on IR&CSA* for further general guidance).

R.7.4.3.1 Non-human data on acute toxicity

R.7.4.3.1.1 Non-testing data on acute toxicity

Non-testing data can be provided by the following approaches:

a) structure-activity relationships (SARs) and quantitative structure-activity relationships (QSARs), collectively called (Q)SARs, and expert systems

b) read-across and grouping

(Q)SAR models

Compared with some other endpoints, there are relatively few (Q)SAR models and expert systems capable of predicting acute toxicity. Available approaches have been reviewed in the literature (Cronin *et al.*, 1995, 2003; Lessigiarska *et al.*, 2005; Tsakovska *et al.*, 2006; Fuart Gatnik and Worth, 2010).

(Q)SAR software packages (commercial and free) that include models for the prediction of acute toxicity are: the OECD QSAR Toolbox, HazardExpert, Topkat, CASE Ultra, T.E.S.T, Derek Nexus and ACD/Percepta. Some of the models available from the scientific literature and the aforementioned softwares are described in Appendix R.7.4-2.

On the basis of these reviews, the following conclusions can be made:

i) the relatively small number of models for *in vivo* toxicity is related to the nature of the endpoint – acute toxicity measurements are usually related to whole body phenomena and are therefore very complex. The complexity of the mechanisms involved leads to difficulties in the QSAR modelling process;

ii) most QSAR models identify hydrophobicity as a parameter of high importance for the modelled toxicity. In addition, many models indicate the role of the electronic and steric effects;

iii) most literature-based models are restricted to single classes of substances, such as phenols, alcohols, anilines. Models based on more heterogeneous data sets are those incorporated in the expert systems.

Read-across and grouping

Read-across/chemical categories are described in Sections R.6.1 and R.6.2 of Chapter R.6 of the *Guidance on IR&CSA*. The scientific basis for building grouping arguments and read-across cases were revisited in the second version of the OECD Guidance on grouping of chemicals (OECD, 2014). More detailed advice on the assessment of read across can be found in ECHA's Read-Across Assessment Framework – RAAF (see http://echa.europa.eu/en/support/grouping-of-substances-and-read-across). Softwares such as the OECD QSAR Toolbox can be used to find data for analogues and support read-across cases.

R.7.4.3.1.2 Testing data on acute toxicity

In vitro data

There are currently no *in vitro* tests that have been officially adopted by the EU or OECD for the (regulatory) assessment of acute toxicity.

- 3T3 Neutral Red Uptake (3T3 NRU) Cytotoxicity Assay:

Based on the validation study to assess the predictive capacity of the 3T3 NRU *in vitro* cytotoxicity test, EURL ECVAM issued a recommendation concerning the validity and limitations of this *in vitro* test (EURL ECVAM, 2013). This recommendation is based on the views expressed by the EURL ECVAM Scientific Advisory Committee (ESAC) (see https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/3t3-nru-recommendation).

According to the validation study, the 3T3 NRU test method shows a high sensitivity (ca 95%) and, consequently, a low false negative rate (ca 5%) when employed in conjunction with a prediction model to distinguish potentially toxic versus non-toxic (i.e. classified versus non-classified) substances. Therefore, substances found to be negative in this test would most likely not require classification for acute oral toxicity based on a cut-off value of >2000 mg / kg bw.

Following the provisions of the REACH Regulation, and in particular those contained in Annex XI, data from the 3T3 NRU test method could be used within a WoE approach to adapt the standard information requirements for acute oral toxicity.

A recommended application and the limitations of the 3T3 NRU test are described in Appendix R.7.4-1.

Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) Assay:The CFU-GM assay (DB-ALM Protocol n°101, see http://ecvam-dbalm.jrc.ec.europa.eu/beta/) has been validated by EURL ECVAM to predict anticancer agents induced myelotoxicity in humans (ESAC, 2006). Its applicability to chemicals' induced toxicity has been further assessed (Cerrato *et al.*, 2009) and. if sufficiently validated and suited to the purpose of assessment of acute toxicity, this assay could be included in a WoE approach (Prieto *et al.*, 2013).

Animal data

Data may be available, particularly for phase-in substances, generated from a wide variety of animal test guideline studies, which give different direct or indirect information on the acute toxicity of a registered substance, e.g.:

- OECD TG 401 (EU B.1) Acute Oral Toxicity (method deleted from the OECD Guidelines for testing of chemicals and from Annex V to Directive 67/548/EEC[1])
- OECD TG 420 (EU B.1 bis) Acute oral toxicity – Fixed dose procedure
- OECD TG 423 (EU B.1 tris) Acute oral toxicity – Acute toxic class method
- OECD TG 425 Acute oral toxicity – Up-and-down procedure (updated in 2008)
- OECD TG 402 (EU B.3) Acute dermal toxicity
- OECD TG 403 (EU B.2) Acute inhalation toxicity
- OECD TG 433 "Acute Inhalation Toxicity, Fixed Dose Procedure" (updated in 2009);
- OECD TG 436 "Acute Inhalation Toxicity, Acute Toxic Class Method" (adopted in 2009);
- OECD TG 434 "Acute Dermal Toxicity, Fixed Dose Procedure";
- ICH compliant studies;
- Mechanistic and toxicokinetic studies;
- Studies in non-rodent species.

[1] Existing OECD TG 401 (EU B.1) data would normally be acceptable but testing using this deleted method must no longer be performed.

Some repeated dose toxicity (RDT) studies can also give useful information. Guidance on how to use information from a sub-acute oral toxicity study is given in Appendix R.7.4-1

Traditionally, acute toxicity tests on vertebrate animals have used mortality as the main observational endpoint, usually in order to determine the LD_{50} or LC_{50} values. These values were regarded as key information for hazard assessment and as supportive information for risk assessment.

However, derivation of a precise LD_{50} or LC_{50} value is no longer considered essential. Indeed, some of the current standard acute toxicity test guidelines, such as the fixed dose procedures (OECD TG 420/ EU B.1 bis and OECD TG 433), use signs of non-lethal toxicity. These test methods should be preferred as they present advantages over the other guidelines in terms of animal welfare.

Generic definitions of "Evident toxicity" and clinical signs indicative of "predictable death" are been given in Annex 1 of the OECD TG 420.

Published and unpublished toxicological or general data

In addition to the current regulatory *in vivo* methods, acute toxicity data on animals may be obtained by conducting a literature search and reviewing all available published and unpublished toxicological or general data, and the official/existing acute toxicological reference values. For more extensive general guidance see Section R.3.1 of Chapter 3 of the *Guidance on IR&CSA*.

Utilising all the available information from sources such as those above, a WoE approach should be undertaken to maximise the use of existing data and minimise the commissioning of new testing. A WoE adaptation, specific to substances of low toxicity is described (and instructed for) in Appendix R.7.4-1.

R.7.4.3.2 Human data on acute toxicity

Acute toxicity data on humans may be available from:

- Epidemiological data identifying hazardous properties and dose-response relationships;
- Routine data collection, poisons data, adverse event notification schemes, coroner's report;
- Biological monitoring/personal sampling;
- Human kinetic studies – observational clinical studies;
- Published and unpublished industry studies;
- National poisoning centres;
- Scientific publications.

The main obstacles to the use of human data are their limited availability and often limited information on levels of exposure (ECETOC, 2004).

R.7.4.3.3 Exposure considerations for acute toxicity

With regard to acute toxicity, exposure considerations are detailed in column 2 of Annex VIII to the REACH Regulation, but not in Annex XI.

If there is only one demonstrated route of exposure, this route must be addressed. Where the potential for human exposure exists, the most likely route, or routes, of exposure should be determined so that the potential for acute toxicity by these routes can be assessed. Determination of the most likely route of exposure will have to take into account not only how the substance is manufactured and handled, including engineering controls that are in place to limit exposure, but also the physico-chemical properties of the substance, for instance, whether the substance is a solid or liquid, the particle size and proportion of respirable and inhalable particles, vapour pressure and log P.

R.7.4.4 Evaluation of available information on acute toxicity

The detailed generic guidance provided in Chapter R.4 of the *Guidance on IR&CSA* on the process of judging and ranking the available data for its adequacy (reliability and relevance), completeness and remaining uncertainty is relevant to information on acute toxicity.

R.7.4.4.1 Non-human data on acute toxicity

R.7.4.4.1.1 Non-testing data on acute toxicity

Physico-chemical properties[2]

It may be possible to infer from the physico-chemical characteristics of a substance whether it is likely to be corrosive or absorbed following exposure by a particular route and, produce acute toxic effects. Physico-chemical properties may be important in the case of the inhalation route (vapour pressure, mean mass aerodynamic diameter (MMAD)[3], log K_{ow}), determining the technical feasibility of the testing and acting upon the distribution in the airways in particular for *local-acting substances*. Indeed, some physico-chemical properties of the substance or mixture could be the basis for waiving testing. In particular, it should be considered for low volatility substances, which are defined as having vapour pressures $<1 \times 10^{-5}$ kPa (7.5×10^{-5} mmHg) for indoor uses, and $<1 \times 10^{-4}$ kPa (7.5×10^{-4} mmHg) for outdoor uses. Furthermore, inhalable particles are capable of entering the respiratory tract via the nose and/or mouth, and are generally smaller than 100 µm in diameter. Particles larger than 100 µm are less likely to be inhalable. In that way, particular attention should be driven on results of aerosol particle size determination.

In particular, for substances in powder form, particle size of the material decisively influences the deposition behaviour in the respiratory tract and potential toxic effects. Particle size considerations (determined by e.g. granulometry testing, OECD TG 110) can be useful for:

- selecting a representative sample for acute inhalation toxicity testing;
- assessing the respirable and inhalable fractions, preferably based on aerodynamic particle size;

[2] Refer also to Appendix R.7.4-1 and to Tables R.7.12-1 to R.7.12-6 in Section R.7.12 of Chapter R.7c of the *Guidance on IR&CSA.*

[3] Forms or physical states in which the substance or mixture is placed on the market and in which it can reasonably be expected to be used must be taken into consideration for classification.

- justifying derogations from testing, for instance, when read-cross (or chemical grouping approach) data can be associated with results from particle size distribution analyses (see Section R.6.2 of Chapter R.6 of the *Guidance on IR&CSA*).

Physico-chemical properties are also important for determination of the potential of exposure through the skin, for example, log K_{ow}, molecular weight and volume, molar refraction, degree of hydrogen bonding, melting point (Hostýnek, 1998).

(Q)SAR

Several (Q)SAR systems are available that can be used to make predictions about, for example, dermal penetration or metabolic pathways. However, these systems have not been extensively validated against appropriate experimental data and it has not been yet verified if the results genuinely reflect the situation *in vivo*. That is why the modelled data can be used for hazard identification and risk assessment purposes only as part of a WoE approach.

These approaches can be used to assess acute toxicity if they provide relevant and reliable (adequate) data for the substance of interest. Guidance on how to assess the relevance and reliability of non-testing data is provided in the general guidance on (Q)SARs in Section R.6.1 and on grouping approaches in Section R.6.2 of Chapter R.6 of the *Guidance on IR&CSA*. Non-testing methods should be documented according to the appropriate reporting formats (see Sections R.6.1.9 and R.6.2.6). In the case of (Q)SARs and expert systems, a detailed description of available models is provided in the JRC QSAR Model Database (http://qsardb.jrc.it/).

The complexity of the acute toxicity endpoint (possibility of multiple mechanisms) is one of the reasons for limited availability and predictivity of QSAR models. In the absence of complete validation information, available models could be used as a part of the WoE approach for hazard identification and risk assessment purposes after precise evaluation of the information derived from the model.

Evaluation of the validity of the method

An evaluation of model validity according to the OECD principles should be available, as described in Section R.6.1, using the QSAR Model Reporting Format (QMRF).

Evaluation of the reliability of the individual prediction

The reliability of individual (Q)SAR predictions should be evaluated, as described in Section R.6.1, using the QSAR Prediction Reporting Format (QPRF).

Read-across and grouping

Generic guidance on the application of grouping approaches is provided in Section R.6.2 of Chapter R.6 of the *Guidance on IR&CSA* and in the RAAF document (see http://echa.europa.eu/en/support/grouping-of-substances-and-read-across). The RAAF document describes the assessment of the suitability of the analogues distinguishing six possible scenarios to build a read-across argumentation. The scenario is determined based on whether the analogues are used alone or in chemical categories, biotransformation and the change in the potency of the effect.

R.7.4.4.1.2 Testing data on acute toxicity

In vitro data

The *in vitro* tests, the 3T3 NRU test in particular, may provide supplementary information, which may be used e.g. to determine starting doses for *in vivo* studies, and to assist evaluation of data from animal studies, especially in identification of species differences. They cannot be used to replace testing in animals completely, but should rather be used in a WoE context.

In vitro data may be useful for predicting acute toxicity in humans providing that the domain of applicability for the test method is appropriate for the class of substances under evaluation and a range of test concentrations have been investigated that permit calculation of an IC_{50} (inhibitory concentration 50%) value. Indeed, on the basis of a preliminary comparison of data, there is the indication that the results of *in vitro* cytotoxicity tests may be more predictive of acute oral toxicity in humans than rat or mouse data. This aspect needs to be further investigated.

Generic guidance is given in Chapter R.4 of the *Guidance on IR&CSA* for judging the applicability and validity of the outcome of various study methods, assessing the quality of the conduct of a study (including how to establish whether the substance falls within the applicability domain of the method and the validation status for the given domain) and aspects such as vehicle, number of duplicates, exposure/ incubation time, GLP-compliance or comparable quality description.

Animal data

Acute toxicity tests on animals have primarily used mortality as the main observational endpoint, usually in order to determine LD_{50} or LC_{50} values, although some of the current standard protocols, such as the fixed dose procedure (OECD TG 420, EU B.1 bis), use evident signs of toxicity in place of mortality. In many cases, there will be little information on the cause of death or mechanism underlying the toxicity, and only limited information on pathological changes in specific tissues or clinical signs, such as behavioural or activity changes.

Many acute toxicity studies on substances of low toxicity are performed as limit tests. For more harmful substances choice of optimum starting dose will minimize use of animals. When multiple dose levels are assessed, characterisation of the dose-response relationship may be possible and signs of toxicity identified at lower dose levels may be useful in estimating LOAELs or NOAELs for acute toxicity. The use of sub-acute oral toxicity studies for the characterisation of acute oral toxicity is described in Appendix R.7.4-1. For local acting substances, mortality after inhalation may occur due to tissue damage in the respiratory tract. In these cases, the severity of local effects may be related to the dose or concentration level and therefore, it might be possible to identify a LOAEL or NOAEL. For systemic toxicity, there could be some evidence of target organ toxicity (pathological findings have to be documented) or signs of toxicity based on clinical observations.

Whichever approach is used in determining acute toxicity critical information needs to be derived from the data to be used in risk assessment. It is important to identify those dose levels which produce signs of toxicity, the relationship of the severity of these with dose and the level at which toxicity is not observed (i.e. the acute NOAEL).

In addition to current available OECD or EU test methods (see Section R.7.4.3), alternative *in vivo* test methods for assessment of acute dermal and inhalation toxicity are in the process for adoption and use for regulatory purposes. Whichever test is used to evaluate acute toxicity on animals, the evaluation of studies takes into account the reliability based on the approach of Klimisch *et al*. (1997) (standardised methods, GLP, detailed description of the publication), the relevance, and the adequacy of the data for the purposes of evaluating the given hazard from acute exposure (for more guidance see

Section R.4.2). The best studies are those that give a precise description of the nature and reversibility of the toxic effect, the number of subjects, gender, the number of animals affected by the observed effects and the exposure conditions (atmosphere generation for inhalation, duration and concentration or dose). The relevance of the data should be determined in describing the lethal or non-lethal endpoint being measured or estimated.

In addition, when several studies results are available for one substance, the most relevant one should be selected; data from others studies that have been evaluated should be considered as supportive data for the full evaluation of the substance.

The classification criteria for acute inhalation toxicity relate to a 4-hour experimental exposure period. If data for a 4-hour period are not available then extrapolation of the results to 4 hours are often achieved using Haber's Law (C.t = k). However, there are limits to the validity of such extrapolations, and it is recommended that the Haber's Law approach should not be applied to experimental exposure durations of less than 30 minutes or greater than 8 hours in order to determine the 4-hour LC_{50} for C&L purposes.

Nowadays a modification of Haber's Law is used ($C^n.t = k$) as for many substances it has been shown that n is not equal to 1 (Haber's Law). In case extrapolation of exposure duration is required, the *n* value should be considered. If this *n* value is not available from literature, a default value may be used. It is recommended to set n = 3 for extrapolation to shorter duration than the duration for which the LC_{50} or EC_{50} was observed and to set n = 1 for extrapolation to longer duration (ACUTEX project, 2006), also taking the range of approximately 30 minutes to 8 hours into account.

Experimentally, when concentration-response data are needed for specific purposes, OECD TG 403 (EU B.2) or the CxT approach could be taken into consideration. The OECD TG 403/(EU B.2 will result in a concentration-response curve at a single exposure duration, the CxT approach will result in a concentration-time-response curve, taking different exposure durations into account. The CxT approach (under consideration for the revision of OECD TG 403) uses two animals per CxT combination and exposure durations may vary from about 15 minutes up to approximately 6 hours. This approach may provide detailed information on the concentration-time-response relationship in particular useful for risk assessment and determination of NOAEL/LOAEL.

R.7.4.4.2 Human data on acute toxicity

When available, epidemiological studies, case reports or information from occupational surveillance may be crucial for acute toxicity and can provide evidence of effects that are undetectable in animal studies (e.g. symptoms like nausea or headache). Nevertheless, the conduct of human studies is not allowed for the purpose of the REACH and CLP Regulations.

Such data could also be useful to identify particular sensitive sub-populations like new born, children, patients with diseases (in particular with chronic respiratory, e.g. asthma, BPOC).

Additional guidance is provided on the reliability and the relevance of human studies because there are no standardised guidelines for such studies (except for odour threshold determination) and these are not usually conducted according to GLP. Such guidance is provided in Section R.4.3.3 of Chapter R.4 of the *Guidance on IR&CSA*.

R.7.4.4.3 Exposure considerations on acute toxicity

Particular attention should be addressed to the potential routes of exposure in humans to select the appropriate testing strategy.

R.7.4.4.4 Remaining uncertainty on acute toxicity

In most cases, remaining uncertainties will exist due to the absence of valid human acute toxicity data, and so appropriate assessment factors should be applied. Toxicokinetic data could help in deriving substance-specific interspecies assessment factors. As acute toxicity testing does not usually include clinical chemistry, haematology and detailed histopathology and functional observations, an additional assessment factor may need to be applied when a NOAEL or LOAEL from these studies is used to derive DNELs (for more guidance on the setting of DNELs for acute toxicity, see Appendix R.8-8 of Chapter R.8 of the *Guidance on IR&CSA*).

R.7.4.5 Conclusions on acute toxicity

R.7.4.5.1 Concluding on suitability for Classification and Labelling

In order to achieve classification and labelling, the criteria set forth in the CLP regulation (Annex I, section 3.1) must be applied. The criteria for classification are based on specific 'cut offs' based on the LD_{50} or LC_{50}.

Ideally, classification and labelling should be achieved using data generated from studies conducted in accordance with officially adopted OECD TGs, or test methods incorporated for the time being into the Test Methods Regulation 440/2008[4]. Such studies will permit identification of the LD_{50}, LC_{50}, the discriminating dose (fixed dose procedures), or a range of exposure where lethality and/or severe toxicity is expected (acute toxic class methods). For materials of low toxicity (no mortalities expected at the upper dose limit) testing is restricted to this dose level (the limit test) and if absence of mortalities is confirmed, classification of the substance with respect to acute toxicity is not required. This option/approach is described in detail in Appendix R.7.4-1.

In the Up-and-Down Procedure (OECD TG 425), where individual animals are dosed sequentially, estimation of the LD_{50} with a confidence interval is possible and this can be used for classification purposes. Data generated in the fixed dose/concentration procedures (OECD TG 420, TG 433 and TG 434 and EU B.1 bis) and the acute toxic class methods (OECD TG 423, TG 436 and EU B.1 tris) are equally sufficient for classification purposes. In the fixed dose/concentration procedures, the discriminating dose is identified as the dose causing evident toxicity but not mortality, and must be one of the four dose levels specified in the test method. Evident toxicity is a general term describing clear signs of toxicity such that at the next highest dose level, either severe pain and enduring signs of severe distress, moribund status or probable mortality can be expected in most animals. In the acute toxic class methods, the range of exposure where death is expected is determined by testing at one or more of the four fixed doses. The OECD and EU guidelines for fixed dose procedure and acute toxic class methods include flow charts that allow conclusions to be drawn with respect to GHS classification. In addition the flow charts in the acute toxic class methods allow identification of LD_{50} or

[4] The Test Methods Regulation is regularly updated to follow the approval of the new OECD Test Guidelines..

LC_{50} cut offs. In the absence of GLP compliant data generated in accordance with OECD or EU methods, all other available information should be considered. Each individual set of data (e.g. a non-GLP study) must be assessed for reliability and relevance as stated in Section R.7.4.4 and any unsuitable data (i.e. that considered unreliable or not relevant) should be disregarded. When experimental data for acute toxicity are available in several animal species, scientific judgement should be used in selecting the most relevant data from among the valid, well-performed tests. When equally reliable data from several species are available, priority should be given to the data relating to the most sensitive species, unless there are reasons to believe that this species is not an appropriate model for humans. If definitive classification and labelling cannot be achieved from any individual source, but multiple sets of data all lead to the same conclusion, then, the WoE approach might be sufficient to classify and a robust proposal detailing this should be put forward (see Appendix R.7.4-1).

Where evidence is available from both humans and animals and there is a conflict between the findings, the quality and reliability of the evidence from both sources shall be evaluated in order to resolve the question of classification. Generally, data of good quality and reliability in humans shall have precedence over other data. However, well designed and conducted epidemiological studies may lack the sufficient number of subjects to detect relatively rare, but nevertheless important, effects. Also, the interpretation of many studies is hampered by difficulties in identifying and taking account of confounding factors. Positive results from well-conducted animal studies are not necessarily negated by the lack of positive human experience but require an assessment of the robustness and quality of both the human and animal data.

If the existing data are contradictory, not concordant or insufficient to reliably determine the appropriate classification and labelling of the substance, additional *in vitro* studies, QSARs, read-across should be considered before conducting any OECD or EU compliant *in vivo* study. In that way *in vitro* data could have a supporting role in a read-across or chemical grouping approach. Study data, which permit an assessment of dose response relationship, should be considered for risk assessment and classification and labelling.

Of particular importance in classifying for inhalation toxicity is the use of well-articulated values in the high toxicity categories for dusts and mists. Inhaled particles with a MMAD between 1 and 4 microns will deposit in all regions of the rat respiratory tract. This particle size range corresponds to a maximum dose of about 2 mg/L. In order to achieve applicability of animal experiments to human exposure, dusts and mists would ideally be tested in this range in rats. The cut off values in the table for dusts and mists allow clear distinctions to be made for materials with a wide range of toxicities measured under varying test conditions.

R.7.4.5.2 Concluding on suitability for Chemical Safety Assessment

For chemical safety assessment, both standard OECD TG/EU test method data and all applicable data considered both reliable and relevant should be used. A quantitative rather than qualitative assessment is preferred to conclude on the risk posed by a substance with regards to acute toxicity dependent on the data available and the potential exposure to the substance during the use pattern/lifecycle of the substance. If quantitative data are not available, the nature and the severity of the specific acute toxic effects can be used to make specific recommendations with respect to handling and use of the substance.

Information on acute toxicity is not normally limited to availability of a LD_{50} or LC_{50} value. Additional information which is important for the chemical safety assessment will be both qualitative and quantitative and will include parameters such as the nature and

severity of the clinical signs of toxicity, local irritant effects, the time of onset and reversibility of the toxic effects, the occurrence of delayed signs of toxicity, body weight effects dose response relationships (the slope of the dose response curve), sex-related effects, specific organs and tissues affected, the highest non-toxic and lowest lethal dose (adapted from ECETOC Monograph No. 6, 1985).

If human data on acute toxicity is available, it is unlikely that this will be derived from carefully controlled studies or from a significant number of individuals. In this situation, it may not be appropriate to determine a DNEL from this data alone, but the information should certainly be considered in the WoE and may be used to confirm the validity of animal data. In addition, human data should be used in the risk assessment process to be able to determine DNEL for particular sensitive sub-populations like new-born, children or those in poor health (patients).

For more extensive guidance on the setting of DNELs for acute toxicity, see Appendix R.8-8 of Chapter R.8 of the *Guidance on IR&CSA*.

The anticipated effects from physico-chemical properties and bioavailability data on the acute toxicity profile of the substance must also be considered in the Chemical Safety Assessment.

R.7.4.5.3 Information not adequate

A WoE approach, comparing available adequate information with the tonnage-triggered information requirements by REACH, may result in the conclusion that the requirements are not fulfilled.

In absence of data from test guidelines or equivalent methods, data from other endpoints could be helpful for the determination of acute toxicity potential. For example, data could be provided by subchronic toxicity or neurotoxicity studies, as in general the design of these studies includes a pilot study to determine dose of departure for the main test. In order to proceed with further information gathering the following testing strategy can be adopted.

R.7.4.6 Testing and assessment strategy for acute toxicity

R.7.4.6.1 Objective / General principles

The main objective of this Testing and assessment strategy is to provide advice on how the REACH Annexes VII and VIII information requirements for acute toxicity can be met using the most humane methods. If the strategy is followed, the information generated will be sufficient to make a classification decision with respect to acute toxicity hazard and may provide data for the risk assessment and DNEL derivation. In addition, assessment of acute toxicity may provide information that is valuable for the conduct of repeated dose toxicity studies, such as identification of target organ toxicity and dose selection.

By adhering to the criteria outlined in the previous chapters, informed decisions may be made on whether sufficient data already exist to cover the objectives, or whether further testing is required.

If further testing is deemed necessary, the use of the most appropriate study in accordance with the REACH Regulation is considered rather than a *one study fits all*

approach. An overarching principle is that all data requirements are met in the most efficient and humane manner so that animal usage and costs are minimised.

R.7.4.6.2 Preliminary considerations

The standard information requirements for acute toxicity under the REACH Regulation are given in Section R.7.4.2.

According to REACH, acute toxicity studies should not be conducted if a substance is known to be corrosive. However, if there are health concerns regarding exposure to non-corrosive concentrations, then acute toxicity assessment may be considered appropriate. In such cases, a specific protocol should be developed as standard LC_{50} or any other *in vivo* acute toxicity testing cannot be performed. For example, *in vitro* data on basal cytotoxicity could be used to establish the most appropriate range of concentrations to be tested.

Regardless of tonnage level, before any testing is triggered, careful consideration of existing toxicological data, exposure characteristics and current risk management procedures is recommended to ascertain whether the fundamental objectives of the strategy have already been met. This consideration should take account of discussions that have taken place under other regulatory schemes, such as CLP, BPR, including earlier regulatory schemes such as the Existing Substances Regulation (EEC) No 793/93, and the EU hazard classification scheme. If it is concluded that further testing is required, then a series of decision points are defined to help shape the scope of an appropriate testing program.

The following four-stage process has been developed for clear decision-making:

- **Stage 1**: gather existing information according to Annex VI;
- **Stage 2**: consider information needs according to the relevant Annex(es) VII to X;
- **Stage 3**: identify data gaps (and adequacy of all available data for classification and labelling and/or risk assessment, or to fulfil the criteria for waiving);
- **Stage 4**: generate new data / propose testing strategy.

R.7.4.6.3 Testing strategy for acute toxicity (see Figure R.7.4–1)

Stage 1. Gathering of existing information

The starting point of the strategy is the review of existing data (e.g. human or animal data, physico-chemical properties, (Q)SARs, *in vitro* test data). For non-corrosive substances, the results of skin and eye irritation and skin sensitisation studies (Annex VII) may provide useful information on the potential for systemic toxicity.

In the ITS, all existing human and test data (e.g. from clinical reports, poisoning cases, animal studies, corrosivity, physico-chemical properties) should be considered. Some information from the existing data e.g. *in vitro* studies (*de novo in vitro* basal cytotoxicity and dermal penetration studies), systemic effects observed in other studies, route of human exposure, physico-chemical properties, dermal or respiratory toxicity of structurally-related substances, might primarily be used for the selection of either an acute *in vivo* inhalation test or an acute *in vivo* dermal test. No specific reference is made to valid (Q)SAR models/approaches or to valid *in vitro* methods, but such data should be assessed when available or generated.

Section R.7.4.3 presents a detailed discussion of the sources that may provide relevant information for the assessment of acute toxicity.

Stage 2. Considerations on information needs

A detailed evaluation of the existing information collated in Stage 1 is conducted to allow an informed decision on the testing needs to fulfil the REACH requirements. It is important to ensure that the available data are relevant and reliable to fulfil these requirements.

It should be noted that if a substance is predicted to be corrosive then further consideration should be given as to whether or not an acute oral test can be justified (in particular in relation with animal welfare considerations). Justifications for conducting a study must be provided in order to minimise the animal use. If the substance is considered likely to be corrosive, no acute toxicity testing should normally be conducted (see Section R.7.4.6.2). Where information on corrosivity is not available then *in vitro* corrosivity tests should be conducted.

The standard information requirements for acute toxicity under the REACH regulation are given in Section R.7.4.2.

When acute toxicity via a second route is required, the choice of the second route (dermal or inhalation) depends on the nature of the substance and the likely route of human exposure. However, information on only one route of exposure may be sufficient and justified (based on physico-chemical, toxicokinetic or human data and review of all possible exposure scenarios; for example with gases only inhalation route could be evaluated as no relevant human exposure may occur by oral or dermal route; for liquid with high viscosity, no testing by inhalation route should be conducted).

If human exposure is possible via inhalation, or if physico-chemical properties indicate that such exposure may occur, then testing via this route for acute toxicity should be conducted. Data from skin/eye irritation, skin sensitisation and acute oral toxicity should be used as indicators to help testing via inhalation (for example, substance with only potential local toxicity; choice of exposure concentrations). If no systemic effects are shown during acute oral testing, then the requirement to conduct inhalation testing should be considered on a case-by-case basis.

Consideration of the need for assessment of acute dermal toxicity should be given if the inhalation route is not considered appropriate. In some cases, it may be possible to draw conclusions about the potential for acute dermal toxicity without further testing, on the basis of the data available from acute oral toxicity and/or dermal absorption studies. Evidence for the potential of high dermal absorption should be considered on a case-by-case basis taking into account physico-chemical properties e.g. Log Kow, water solubility, molecular weight and melting point of the substance. Testing for acute dermal toxicity is indicated if:

- Systemic toxicity is observed in skin/eye irritation and/or skin sensitisation studies;
- Death is observed in an acute oral toxicity test and there is potential for dermal absorption;
- Systemic toxicity is observed in an acute oral toxicity test and there is potential for high dermal absorption (determined following e.g. OECD TG 428, EU B.45);
- There is the potential for high dermal exposure (case-by-case basis).

Stage 3. Identification of data gaps / adequacy of data

The purpose of this step is to identify what additional information is required in order to classify the substance and to perform a risk assessment. In case the available data suggest that the substances is of low toxicity, the WoE-based adapation given in Appendix R.7.4-1 should be considered.

The available information may include data generated using study protocols that differ from the standard regulatory tests. The evaluation should include whether the available information meets or exceeds the data requirements from standard regulatory study protocols. Therefore it may be possible that the tonnage-driven minimum needs can be met through combined data obtained from several sources.

At this stage, it is also necessary to verify if the available information is adequate for hazard characterisation. For this process, all relevant information should be taken into account in a weight of evidence assessment. Quantitative data on the dose response relationship for the critical toxicological effects and/or estimations of the either the LC_{50}/LD_{50} values or the Discriminating Dose will be important for assessing the hazard classification and can be used in the risk assessment. Information from testing for other toxicological endpoints (e.g. repeated dose toxicity) may also be useful for the risk assessment (see also Appendix R.8-8 of Chapter R.8 of the *Guidance on IR&CSA*). Mathematical modelling should be considered for estimating a threshold exposure level (e.g. benchmark dose), as an alternative to generating additional *in vivo* data.

If the data and subsequent decisions are deemed consistent with an adequate hazard characterisation and are sufficient to classify the substance or to conduct a risk assessment, then no further testing for acute toxicity is recommended.

In some cases, the substance may be excluded from acute toxicity testing if it does not appear as scientifically necessary (Annex XI). This might be the case for example if:

- A WoE analysis demonstrates that the available information is sufficient for an adequate hazard characterisation and the exposure to the substance is adequately controlled;
- The substance is not bio-available via a specific route and possible local effects are adequately characterised (example, no dermal absorption for dermal route);
- For inhalation route, no testing is required if it is not technically possible to generate a testing atmosphere, because the vapour pressure or the particle is very low.

Finally, the conclusion that no further testing is required may be reached when the data meet the requirements for classification for toxic effects or if the substance has already been classified for acute toxic effects.

Where evidence is available from both humans and animals and there is a conflict between the findings, the evidence should be evaluated towards understanding the toxicological basis for these divergent findings. Issues relating to the quality and reliability of the data should also be taken into account. Generally, data of good quality and reliability in humans shall take precedence over other data. However, well-designed and conducted epidemiological studies may lack a sufficient number of subjects to detect relatively rare but still significant effects, to assess potentially confounding factors. Positive results from well-conducted animal studies are not necessarily negated by the lack of positive human experience but require an assessment of the robustness and quality of both the human and animal data.

If the remaining data are contradictory, not concordant or insufficient to determine reliably the appropriate classification and labelling of the substance, additional *in vitro*

studies, QSARs, read-across should be considered before conducting any OECD compliant *in vivo* study. Study data, which permit an assessment of dose response relationship, should be considered particularly valuable for risk assessment purposes.

Stage 4. Generation of new data / proposal for testing strategy

If sufficient data for risk assessment and classification purposes are already available, no further testing will be required. If data gaps need to be filled, new data shall be generated (Annexes VII and VIII to the REACH Regulation). Due to animal welfare considerations, new tests on animals should only be performed as a last resort when all other sources of information have been exhausted.

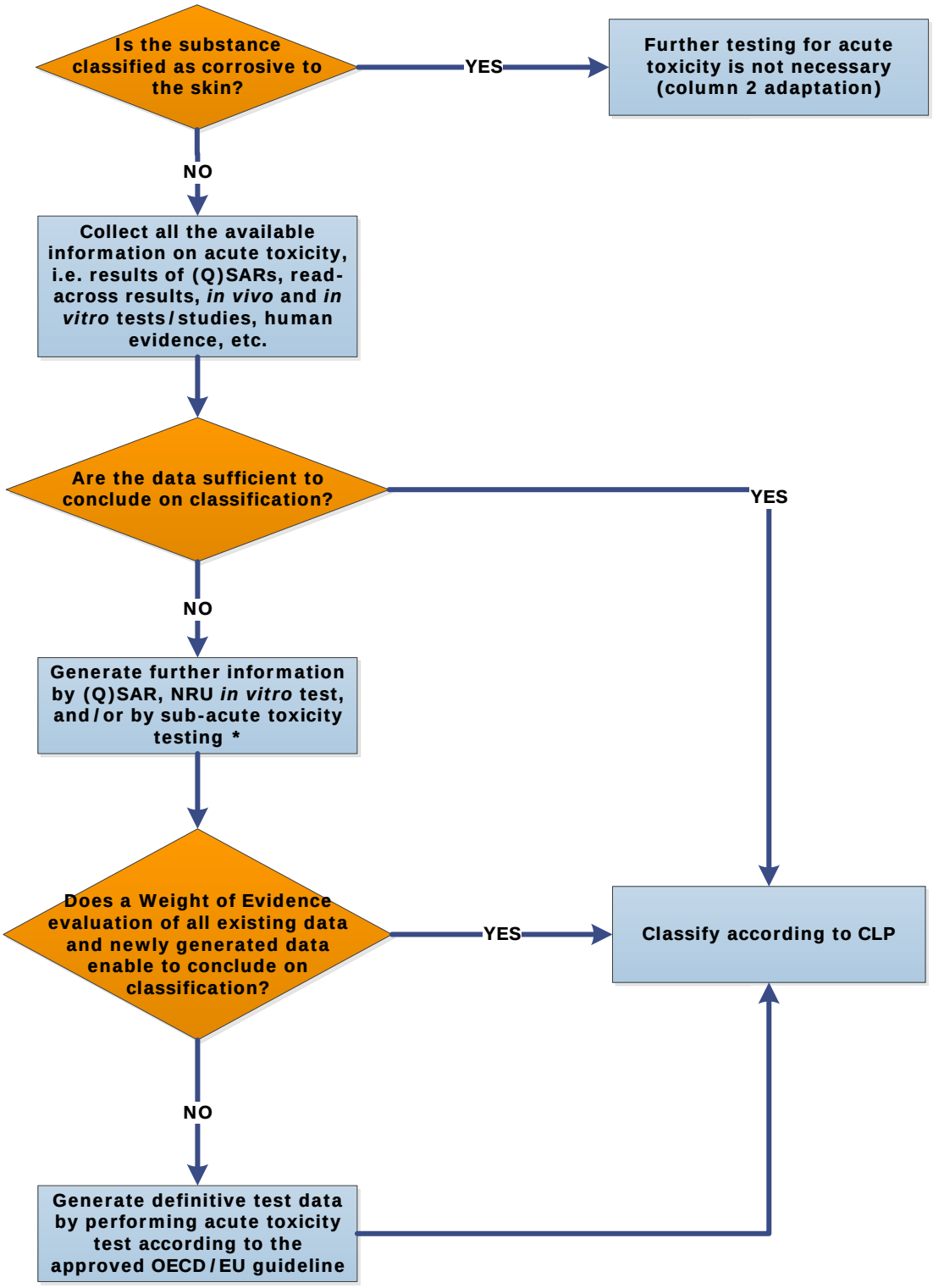
The standard OECD guidelines should normally be used as these provide the necessary information on acute toxicity hazard in a way that balances the need to protect human health with animal welfare concerns (see Section R.7.4.3 and the above guidance for Stage 3).

The route of exposure to be used for acute toxicity evaluation depends on the nature of the substance (e.g. gas or not, molecular weight, log K_{ow}) and should reflect the most likely route of human exposure. If any specific human exposure may be identified, further testing for risk assessment should be considered as proposed in REACH Annex VIII, Section 8.5. If any human exposure by inhalation is identified, then the testing strategy by inhalation should be proposed (Figure R.7.4–2).

First considerations should be based on defining the potential of the substance for acute toxicity. For such a question, information may be provided by existing data from SARs, QSARs, chemical categories approaches and available *in vitro* and *in vivo* data. If no potential for toxicity is shown, then no further testing is required and a decision on classification can be taken. Such information may also provide relevant information in risk assessment considerations. This approach, which is based on evidence of low/no acute oral toxicity (without performing the relevant *in vivo* test according to REACH Annex VII, 8.5), should be documented in a WoE analysis as explained in Appendix R.7.4-1. For this specific WoE case, the sub-acute oral toxicity study is crucial and should usually be available in order to reach a definitive conclusion.

Following the general testing strategy, dose selection appears to be an important aspect in order to select the most appropriate starting point. When validated *in vitro* tests are available, these may provide relevant results, and help the dose selection for oral route testing (see Section R.7.4.4.1).

For substances in the ≥10 t/y tonnage band, testing by the dermal route should be considered if a human exposure is identified, or if results from physico-chemical properties and in particular skin irritation/sensitisation tests show any dermal absorption or any systemic toxicity. Depending on such information, dermal testing should be conducted or not following standard protocols (see Section R.7.4.3).



* the sub-acute toxicity study is only required at Annex VIII and above.

Figure R.7.4–1 Testing and assessment strategy for acute toxicity.

A specific testing strategy is proposed for the inhalation route (Figure R.7.4–2). Primary considerations should be based on the in(ability) to generate a suitable atmosphere depending on the physico-chemical properties (for example, low volatility, solid, particle size >100 μm (see also Section R.7.4.4.1). In this situation, no human exposure may be identified and no further testing is required.

Wherever possible, assessment of acute inhalation toxicity should be conducted in accordance with the OECD TG 433 and TG 436 since they have been designed to use less animals than the OECD TG 403 and EU B.2. In addition, OECD TG 433 does not require mortality as endpoint. However, in some circumstances, i.e. if a dose response curve is needed for risk assessment purposes, testing according to OECD TG 403, EU B.2 or the CxT approach may be considered appropriate (see also OECD Guidance Document 39, (OECD, 2009)).

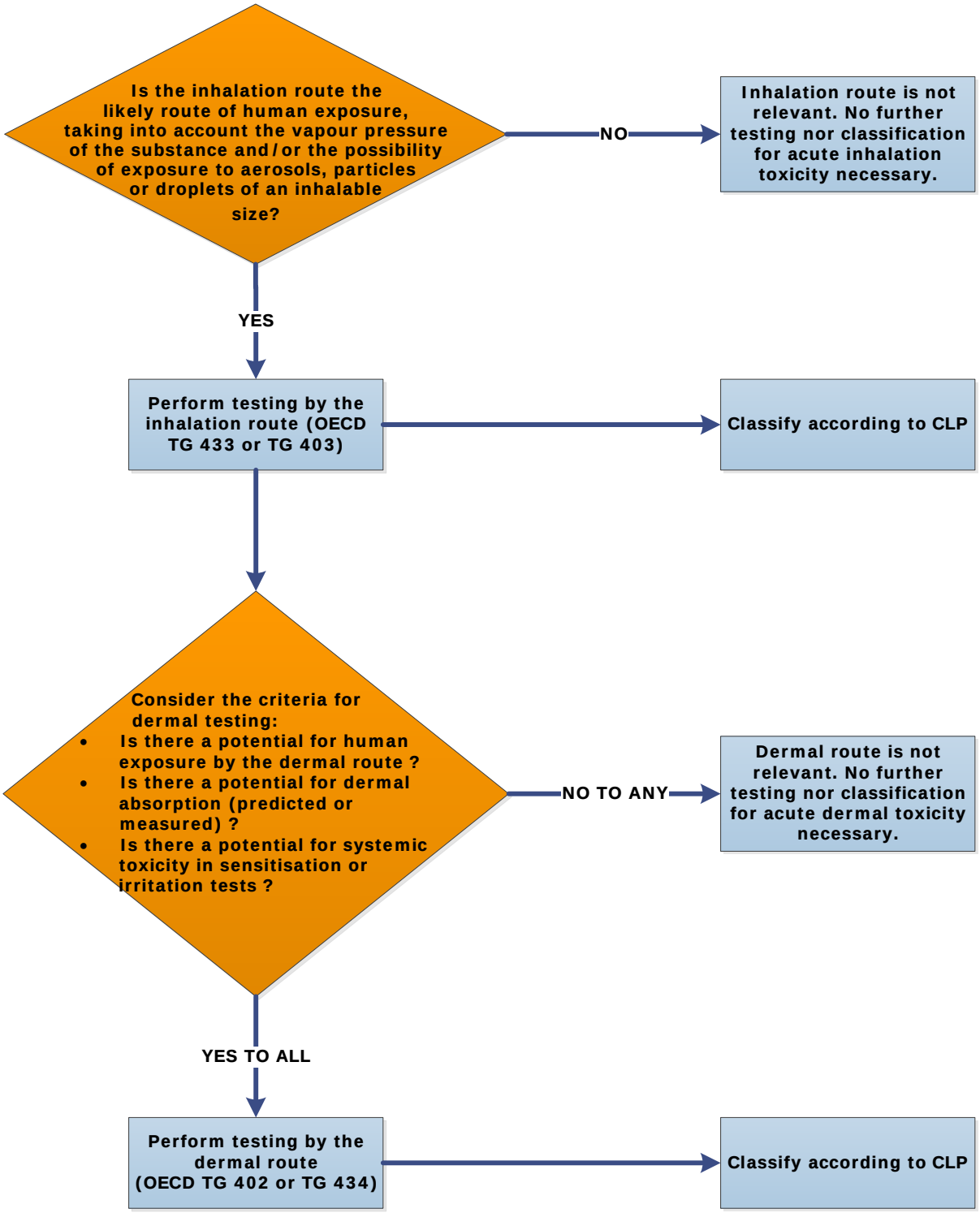


Figure R.7.4–2 Selection of additional routes of exposure for acute toxicity (see also OECD GD 39 (OECD, 2009)

R.7.4.7 References on acute toxicity

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Appendices R.7.4-1 and R.7.4-2 to Section R.7.4

Appendix R.7.4-1 Weight-of-Evidence based adaptation to the standard information requirement for an acute oral toxicity study

The aim of this Appendix is to advise the registrants on how they can perform an *in vivo* acute toxicity study only as a last resort. An *in vivo* acute oral toxicity study can potentially be avoided, if a registrant has relevant data, which is used in a Weight-of-Evidence (WoE) approach. In case the WoE adaptation is acceptable, the registrant is able to avoid unnecessary animal testing pursuant to REACH Articles 13(1) and 25(1). The description of the "elements of evidence", which can be included in a WoE case, is the main scope of this document.

1. Background and scope of the WoE adaptation

Acute oral toxicity is one of the standard information requirements in Annexes VII-X.

An alternative to performing the acute oral *in vivo* acute toxicity test is outlined in this Appendix. Its aim is to reduce the number of animal studies needed, and the cost of testing, by a proposing WoE adaptation, according to REACH Annex XI, section 1.2.

Annex XI specifies several possibilities for adaptation, including e.g. weight-of-evidence (section 1.2), QSAR (section 1.3), and *in vitro* tests (section 1.4), and read-across (section 1.5). In principle registrants may use these adaptation possibilities "in isolation". However, the WoE approach outlined below, and making use of combinations of these elements, is recommended. It is based on ECHA's analysis, and it is more likely to result in an adaptation that can be accepted according to Annex XI, section 1.5.

It is anticipated that many phase-in substances, which will be registered by the 2018 deadline, will have an *in vivo* acute oral toxicity study already available (the estimate is 65%[5]). However many registrants will have to conduct a novel study to meet the acute toxicity information requirement, or to adapt this standard information requirement. It was estimated by ECHA that approximately approximately 550 [6] *in vivo* acute oral toxicity tests could be avoided.

In 2014 EURL ECVAM, part of the Joint Research Centre (JRC) of the European Commission, published a Strategy Document on "Alternative approaches for acute systemic toxicity testing" (http://publications.jrc.ec.europa.eu/repository/handle/JRC90611). EURL ECVAM considered that efforts should be directedtowards (i) the reduction and replacement of animal tests for acute systemic toxicity, and (ii) the refinement of *in vivo* studies, according to the Russell and Burch 3Rs principle. By following the approach proposed in this Appendix, registrants would contribute towards these efforts.

Consideration should be given to the mechanistic basis of acute toxicity and the validation of integrated prediction models. EURL ECVAM proposed to evaluate promising

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[5] From the second Article 117(3) report, published in June 2014: 35% of ca. 5200 substances (to be registered at > 10 tpa, by 2018) are forecast not to have an existing acute oral toxicity study, which represent approximately 1825 studies. It is also assumed that approximately 30% of these substances are of low acute toxicity (ie. where the acute oral LD50 is higher than 2000 mg/kg bw/day). Therefore the use of a waiving possibility of performing an *in vivo* oral acute toxicity testing requirement may have a high impact: if those registrants would follow the alternative approach proposed in this Annex, the number of acute oral toxicity studies necessary for the 2018 registration deadline could be reduced by approximately 550.

[6] ECHA acknowledges that this estimate maybe an underestimation. While the figure holds some uncertainty, ECHA decided to pursue the development of the WoE approach in this Appendix, to enable adaption of the *in vivo* acute oral toxicity study when scientifically justifiable.

components of integrated approaches for testing and assessment (IATA), including the better use of existing alternative methods, such as mechanistically relevant *in vitro* assays. Furthermore, according to EURL ECVAM, information on repeated dose toxicity might be useful in supporting classification and labelling for acute systemic toxicity.

In addition, in the scientific literature the value of the acute toxicity test has been discussed and prediction models based on sub-acute toxicity data or *in vitro* cytotoxicity tests that may replace *in vivo* acute toxicity studies, have been developed (Creton *et al.*, 2010; Chapman *et al.*, 2010; Indans *et al.*, 1998; Kinsner-Ovaskainen *et al,*. 2013; Robinson *et al.*, 2008; Siedle *et al.*, 2011; Bulgheroni *et al.*, 2009).

The scope of the WoE based adaptation outlined below is the following:

- The WoE approach is mainly meant for substances to be registered at **Annex VIII** tonnage level and above (i.e. registrations at >10 tpa), for which an oral sub-acute toxicity study (OECD TG 407) or the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD TG 422) is required[7].
- The WoE approach is intended for substances of low acute toxicity, i.e. for substances with an $LD50_{oral}$ expected to be greater than 2000 mg/kg bw;

These and other limitations are described below in the specific chapters in more detail.

The background and rationale of this guidance for a WoE-based adaptation for the acute oral toxicity study is based on the following:

- There are several initiatives and proposals made by the scientific community suggesting that relevant information on the acute oral toxicity can be obtained without performing the standard *in vivo* test.
- In 2015, the JRC launched a survey aimed to explore waiving opportunities for acute systemic toxicity testing. One particular goal was to find out if experts from different fields (pharmaceutical, chemical industry etc.) may have any experience with using data from repeated-dose toxicity studies to predict acute systemic effects. From the responses obtained it became evident that some companies have in fact tried to predict the acute effects from repeated dose studies (personal communication, JRC(1), 2015).
- Several hundreds of *in vivo* studies can potentially be replaced with the WoE approach.

There are several types of studies and information that can be used in the characterisation of the acute oral toxicity of a substance. The types of information, which are presumably of high value in the prediction of the acute oral toxicity, have been included in this Appendix.

The non-prescriptive WoE approach outlined below should consist of more than one of the following elements of evidence[8], and has to include in any case the 28-day repeated dose toxicity study, as the most valuable and essential part of the WoE approach proposed.

[7] The type of adaptation described below could be used, independently of the tonnage band, in case a sub-acute toxicity study is available

[8] The requirement of obtaining and reporting more than one piece of evidence within the WoE follows from the provisions of REACH Annex XI, 1.2.

1. Results of a 28-day repeated dose toxicity study via oral route (the sub-acute study);
2. Results of an "enhanced" dose range finding (DRF1) study supplemented with relevant clinical observations during the first day of dose administration, which will provide valuable information. DRF1 is expected to be performed prior to the main sub-acute oral toxicity study;
3. Data from an NRU *in vitro* study for cytotoxicity (or equivalent); according to the ECVAM recommendation (EURL ECVAM, 2013). The NRU study predicts well substances of low acute oral toxicity;
4. (Q)SAR results which may provide information for the acute oral toxicity;
5. Data on such physico-chemical properties of the substance, which inform of the bioavailability or the reactivity of the substance, and/or which can contribute to the assessment scheme and/or to the grouping approach; and
6. Other supportive evidence, such as, justified read-across information, results from mechanistic and/or tissue-based *in vitro* studies, e.g. addressing neurotoxicity or human data.

These elements of evidence, which are addressed in detail in the next sections, can be examined and considered by the registrants to adapt the standard information requirement of the oral *in vivo* acute toxicity test for their substances.

This Appendix will also provide guidance on how to obtain and assess these different elements of evidence. Finally, two "decision-trees" for the WoE assessment, with different starting elements are outlined in figures 1 and 2 below.

2. Prediction of acute oral toxicity based on the results of a sub-acute oral toxicity study

2.1. Introduction

The WoE approach for the Annex VIII substances with tonnage > 10 tpa has to include data on oral sub-acute toxicity. An analysis initiated by JRC (personal communication, JRC(2), 2013) and then continued by ECHA (see section 2.2.) has shown that, for **substances of low toxicity**, the prediction of **acute oral toxicity** classification can be based on the data from oral **sub-acute studies** in most cases. In particular, the non-classification for oral acute toxicity (i.e. the substance is not to be classified if the LD_{50} is above 2000 mg/kg) can be correctly predicted based on the results of oral sub-acute studies, when the NOAEL was at or above 1000 mg/kg bw.

In this document, the term "low toxicity" is used for substances which have an $LD50_{acute,oral}$ greater than 2000 mg/kg bw and a $NOAEL_{subacute,oral}$ of 1000 mg/kg bw or greater, derived from a repeaded dose toxicity (RDT) study with a duration of at least 28 days.

A quantitative correlation between acute oral toxicity and sub-acute oral toxicity across the whole range of toxicity (i.e. from low toxic to severely toxic substances, $LD_{50} \leq 2000$ mg/kg bw) was also examined, but the results have not been promising.

Therefore the scope of the present WoE approach is explicitly for the substances of **low toxicity**, and relies on "limit test" dose for repeated dose toxicity studies (i.e. NOAEL > 1000 mg/kg, bw) and the classification threshold applied for the acute oral toxicity in the EU (i.e. >2000 mg/kg).

2.2 ECHA analysis of data submitted under REACH

The data used for this analysis were extracted in May 2015 by ECHA from the whole REACH registration database from sections 7.2.1 (Acute toxicity: oral) and 7.5.1 (Repeated dose toxicity: oral) of the IUCLID dossiers.

A preliminary set of filters was used to select **relevant** experimental data:

- "Test material identity same as registered substance" = "yes"
- "study type" = "experimental result" (to select only experimental data and to exclude other study types such read-across or QSAR results)
- Reliability score = "1" or "2"
- An additional filter was used to select the studies performed according to the relevant OECD/EU guidelines:
    - OECD TG 401 (LD50 only), 420, 423, 425; EU Method B. 1 (bis and tris) for acute toxicity
    - NOAEL or NOEL OECD TG 407, 422 for sub-acute, excluding results expressed in ppm.
- Another filter was used to select only dossiers containing **relevant studies in both 7.2.1 and 7.5.1 sections**.

As a result,1453 registration dossiers were selected.

In the remaining registration dossiers, other routes of administration (often inhalation) have been used for the acute and/or sub-acute toxicity tests, or one of these studies has been adapted, e.g. by using information on an analogue substance (i.e. read-across adaptation). Hence no study record(s) in the IUCLID dossier could be used for this analysis.

ECHA then refined the data set as follows:

- exclude sub-acute studies reporting a NOAEL < 1000 mg/kg bw
- If a range was given for a single study, the lowest value was selected
- If the registrant submitted more than one relevant study per endpoint, the study resulting in the lowest LD50 value and/or lowest NOAEL value was selected;

Furthermore, the information on the identity of the test material was checked in order to exclude cases where another substance than the registered substance could have been tested (i.e. "hidden" read-across).[9]

To summarise, the data included in the final prediction model include dossiers with:

- Relevant acute oral and sub-acute oral toxicity /screening study tests[10], and
- Sub-acute oral toxicity study resulted in a NOAEL at or above 1000 mg/kg bw.

Please note that registrant self-classification was not considered.

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[9] With the term "hidden read-across" ECHA refers to studies marked as "experimental results" where "identity of the test material same as registered substance" is ticked, but the identifiers provided in the test material identifiers table refer to a substance different from the registered one.

[10] According to the relevant OECD guidelines, rats and mice are the preferred species. With very few exemptions, the studies used for this "prediction model" were made with these species.

Substances in 442 dossiers fulfil the above criteria. In addition, all except ten dossiers gave an acute oral toxicity study with an LD50 higher than 2000 mg/kg bw. These cases were manually analysed and are explained in the separate Excel file.

In conclusion, this "prediction model" based on sub-acute toxicity data is the core element of the WoE approach.

2.3. Conclusion on the use of an existing sub-acute oral toxicity study to adapt the acute oral toxicity requirement

Where registrants hold an existing subacute oral toxicity study, the results of which indicate that the substance falls within the scope of this WoE approach, the prediction of the acute oral toxicity potential may be used as an element of a WoE adaptation (pursuant to the REACH Annex XI, 1.2). This approach supports the registrant in fulfilling their obligation under Article 13(1).

Based on this prediction, the registrant may also conclude that the classification and labelling for acute toxicity is not warranted.

Since this prediction focuses on substances of low toxicity, it is important to note the following limitations:

- The WoE cannot be used for any substance where the results of a sub-acute oral toxicity study resulted in a NOAEL below 1000 mg/kg bw. A quantitative analysis made by JRC has shown that the correlation between the sub-acute and acute toxicity across the whole range of NOAELs and LD50 values is poor.
- The WoE approach cannot be proposed, if no sub-acute oral toxicity study (OECD TG 407 or TG 422) has been performed.
- The WoE cannot be used for any substance which requires the GHS classification as "acute toxicity category 5"[11] (i.e. where the $LD50_{acute,oral}$ is higher than 2000 mg/kg bw and lower than 5000 mg/kg bw).

2.4. Use of a novel dose range finding study and of a novel sub-acute toxicity study

When registrants do not hold a (valid) sub-acute oral toxicity study for substances manufactured or imported at tonnage > 10 tpa, they will need to perform a novel study to fulfil the legal requirements at Annex VIII (section 8.6.1).

2.4.1. Dose-range-finding studies (DRF)

Before a novel sub-acute oral toxicity study (OECD TG 407 or OECD 422) is conducted, appropriate doses must be identified. For this purpose, the registrant may either use existing data (screening studies, acute toxicity studies, literature data) or/and perform one or more dose-range-finding studies (DRF).

DRF1

If virtually nothing is known about the substance, the first part of the DRF study (pilot study) may consist of a single administration of one dose to 2 animals and subsequently, depending on the reaction of the animals, with single administrations of lower or higher

[11] The "Acute toxicity category 5" classification may be needed for some countries outside the EU.

doses to additional animals. Thus one gets an impression of the acute toxicity of the substance.

Investigations are normally restricted to cage-side observations for signs of toxicity and gross necropsy in an attempt to identify target organs. Normally, the frequency of observations is several times on the first day, then once or twice a day. The observation period is typically limited to 7 days after administration.

DRF2

Having found the highest dose which will most probably not lead to the death of the animals after repeated administration of the test substance, a second DRF study is usually performed by administering 3 or 4 different doses to groups of 5 males and 5 females (or less) each, once daily, for one week (7 days). Investigations include body weight development, cage-side observations and possibly also clinical observations. The frequency of cage-side observations is normally twice to four times on the first day, then twice a day for 7 days. At the end of the administration period, gross necropsy is performed, but no histopathology or clinical chemistry or haematology is undertaken.

Based on these findings the doses for the main study are selected.

Contribution of DRFs to the WoE

The advantages of using DRF1 as one element in this WoE approach are that (i) only a low number of animals is needed and (ii) that high doses up to 2000 mg/kg may be administered. Furthermore, more frequent observations of the signs of toxicity can be relatively easily arranged for, to obtain valuable information on whether animals dosed with up to 2000 mg/kg bw survive without signs of toxicity.

DRF2 on the other hand, provides data on toxicity after repeated exposure. As the doses may be higher than in the main study, some additional information on acute toxicity may be gained.

The information will be most valuable if animals which are dosed up to 2000 mg/kg bw survived without signs of toxicity. The NOAEL derived from a 7-day toxicity study (DRF2) cannot be used as stand-alone for the sub-acute oral toxicity requirement.

Therefore ECHA recommends that in case they aim to adapt the acute oral toxicity requirement, and to use DRF1 as a part of the WoE, the registrants perform an "enhanced" DRF1.

2.4.2. Enhanced DRF1

To enhance the information provided by the DRF1 tests, the frequency of the clinical observation needs to be adjusted for the first day of DRF1, to the scheme of the acute oral toxicity test guidelines. The observation period should be prolonged to a total of 14 days after the administration of the test substance, so that *"animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days*" (OECD TG 420, Acute Oral Toxicity – Fixed Dose Procedure, Adopted in 2001).

The clinical observations during the enhanced DRF1 (type and level of details) should follow the ones specified in the OECD acute oral toxicity test guidelines (Table 1).

Notes: Registrants are reminded that the DRF1 observations (from the first day mainly) are to be reported also separately and an endpoint study record which covers the DRF1 needs to be submitted under the Acute Toxicity Endpoint in the IUCLID dossier (section 7.2.1). It is essential that documentation of the observations

and the findings made in the enhanced DRF1 are submitted with the registration dossier, as part of the WoE approach.

It is acknowledged that in some CROs, the practice of performing the DRF1 may be different from the one recommended. Furthermore, in some EU Member States, for animal welfare considerations, a dose of 1000 mg/kg cannot be exceeded.. If a short duration of observation (omitting the 14 days recovery period) and the low dose limit (i.e. 1000 mg/kg) are followed in the DRF1, the information obtained from it will be of less value in the context of the WoE adaptation presented here. Where the registrant can choose to perform the DRF1 following the recommendation given below, the results obtained are of higher value in the WoE analysis. (Note that figure 2 may also be applied, when a non-enhanced DRF1 is available. In that case, DRF1 is one of the "Additional elements of evidence within WoE".

Costs of additional steps

Together with CROs' experts, ECHA has estimated that additional costs would be generated from (i) approximately 1-3 hours extra time for observations and recording, and (ii) the housing of the animals for 14 days after administration (as opposed to 7 days). It is therefore anticipated that the cost increase of the enhanced DRF1 study will be limited.

2.4.3. Main study, the sub-acute oral toxicity study

The main study, i.e. the sub-acute oral toxicity study provides data on toxicity after repeated exposures. However, information on acute oral toxicity may also be gained from that study.

The obvious advantage of the main study is that its results will be valuable for the WoE approach, in case the NOAEL is at or above 1000 mg/kg bw/ day (see Chapter 1.0 above). It is noteworthy that the 1000 mg/kg bw dose is not the definite upper threshold for a 28-day repeated dose study and that higher doses can be applied, if deemed useful, e.g. for deriving DNELs.[12]

The schedule of observations and the scope of clinical observations in the acute and sub-acute oral toxicity studies are summarised in Table 1, according to the relevant paragraphs of the relevant OECD test guidelines.

Table 1. Comparison of the general clinical observations (as required by the OECD test guidelines for acute oral toxicity and sub-acute oral toxicity) and proposed schedule of observations in the enhanced DRF1 study.

OECD Test Guideline	Day 1		Days 2-14 (acute and DRF1) Days 2-28 (RDT) Days 2-7 (DRF2)
	30-min	4-hour + periodically until 24-hrs	Daily

[12] The main study (sub-acute oral toxicity study) is understood as resulting from performing the test under the OECD TG 407 or OECD TG 422. Regarding the results of an OECD TG 422 study, it is important that the NOAEL used refers to the maternal/paternal toxicity, and not to the NOEL for developmental effects.

TG 420 (Fixed Dose Procedure), TG 423 (Acute Toxic Class method), TG 425 (Up-and-Down-Procedure)	Animals are observed individually, at least once	Animals are observed individually, with special attention given during the first 4 hours	Animals are observed individually
	Additional observations* necessary if animals continue to display signs of toxicity		
Enhanced DRF1 for TG 407[13]	Animals are observed individually, at least once	Animals are observed individually, with special attention given during the first 4 hours	Animals are observed individually
Repeated dose oral toxicity study, TG 407 or TG 422	General clinical observations at least once a day. Morbidity/ mortality at least twice daily		
DRF2 for TG 407	General clinical observations at least once a day. Morbidity/mortality at least twice daily		General clinical observations at least once a day. Morbidity/ mortality at least twice daily

* (at least) Changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

As part of the OECD TGs 420, 423, 425 and enhanced DRF1 (i.e. during the general clinical observations), *"the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary". "The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed (11).) All observations are systematically recorded, with individual records being maintained for each animal."* In addition *"The principles and criteria summarised in the Humane Endpoints Guidance Document should be taken into consideration (8)…. (Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be humanely killed. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.)" (Reference*: OECD TG 420: Acute oral toxicity study, Fixed Dose procedure, paragraphs 27 and 28, as an example for OECD TGs 420, 423 and 425).

2.5 Conclusion on the use of the novel DRFs and subacute oral toxicity study to adapt the acute oral toxicity requirement

Where a sub-acute oral toxicity study is not available and the registrant generates a novel study, it is recommended that the registrant performs an enhanced DRF1 study as proposed in Table 1. If no acute toxicity is seen in the enhanced DRF1 and if the main sub-acute toxicity study falls within the scope of this WoE approach (i.e. NOAEL > 1000 mg/kg bw), this prediction may be used to justify that the performance of a novel acute oral toxicity test is not scientifically necessary (pursuant to the REACH Annex XI, 1.2). In this case, the two main elements of the WoE are the enhanced DRF1 and the main sub-

[13] Enhancement of the DRF1 means that the observation schedule is the identical to the one in the acute toxicity test and that observation lasts for 14 days.

acute toxicity study. Consequently the registrant can propose to not classify the registered substance for acute oral toxicity (Figure 1 below). This approach also supports the registrant in fulfilling their obligation under Article 13(1).

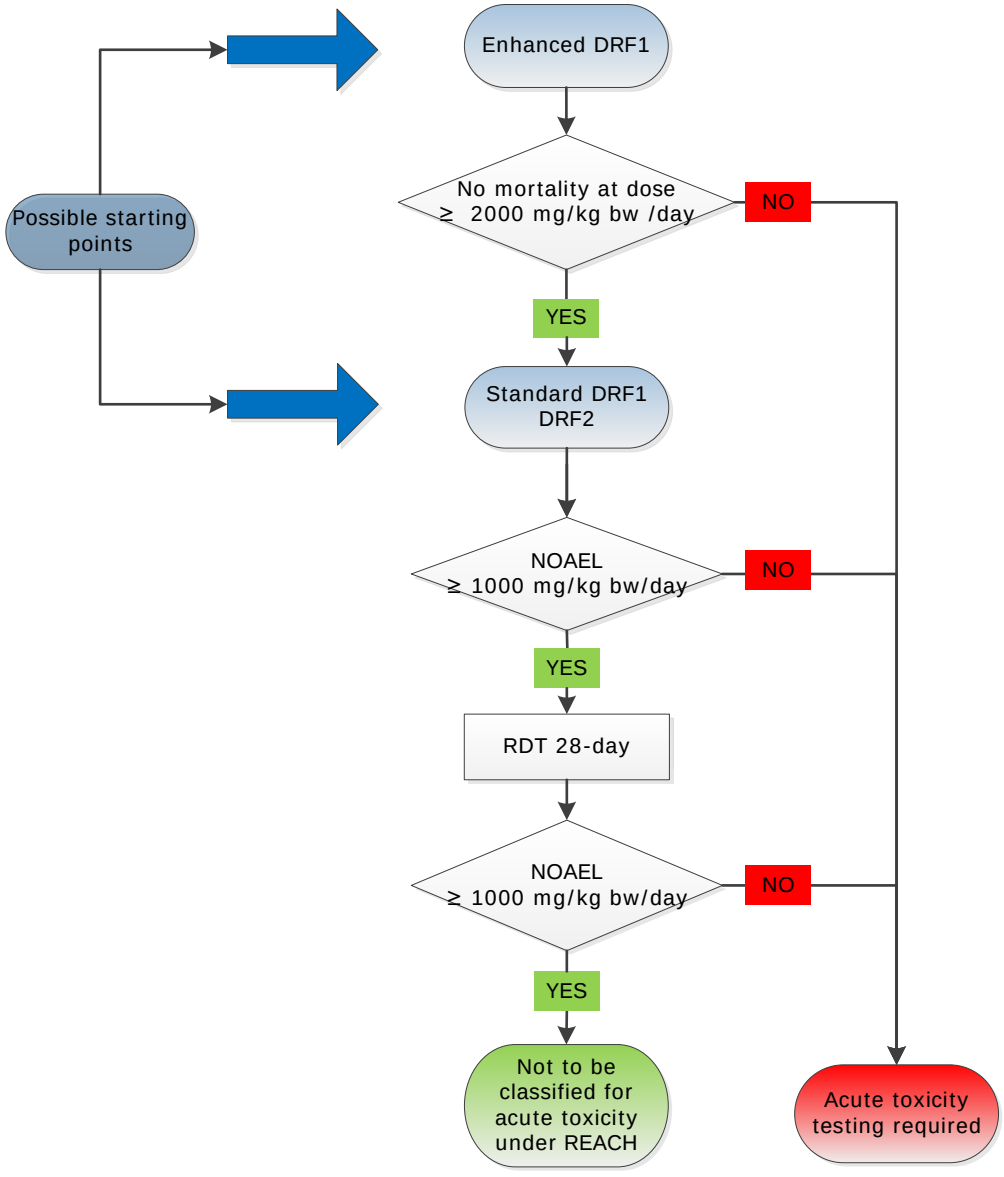

Figure 1: Decision tree to assess whether an *in vivo* acute toxicity test is required, when the registrant has to generate a **novel repeated sub-acute oral toxicity study** and an enhanced DRF is available.

Figure 1 illustrates cases, where data are available from the DRF study(ies) and from a sub-acute oral study and where these data confirm that the substance is of low acute oral toxicity. The Figure also illustrates the situations, where the registered substance would fall outside of the scope defined for this WoE approach and where the *in vivo* acute oral toxicity test will therefore be required.

ECHA acknowledges that registrants may have other data, such as data from a non-enhanced DRF1, data from other in vivo studies in rats where single doses of ≥2000

mg/kg bw or doses of ≥1000 mg/kg bw for several days have been administered, a NRU test (which is currently the only validated *in vitro* cytotoxicity test), a QSAR model or from human evidence, which provide a conclusion consistent with the one obtained from a 28-day subacute study. Registrants may then use those elements of evidence together with the 28-day sub-acute study, instead of using the enhanced DRF1 in their WoE approach (see Figure 2).

It is noteworthy that, currently, the observations made in the DRF studies are not standardised, and therefore ECHA provides relevant instructions in Table 1. Furthermore, the 14-day observation period that is included in an acute oral toxicity test is usually not followed in a DRF study for a sub-acute oral toxicity study. This concurs with the need to generate the "Enhanced DRF1" where the observation period is prolonged.

2.6. Regulatory use of the DRF studies for the WoE approach

If an enhanced DRF1 study (with a limited number of animals, typically 2) is used as part of the WoE approach, at least one of the doses applied should be up to 2000 mg/kg bw (or above in case of old studies). The observations should be made according to the scheme outlined in table 1. The enhanced DRF1 provides information which resembles that obtained from an OECD guideline test for acute oral toxicity, but obtained with less animals than recommended in the test guideline. It can therefore not be a replacement for an OECD guideline study, but maybe a part of the WoE approach. The (enhanced) DRF1 should be used in the registration dossier with an adequate justification of how this information, when taken together with other WoE elements meets the specified REACH information requirement.

When an (enhanced) DRF1 study is used within the WoE, two scenarios may occur:

1. There are **no or only transient** signs toxicity at a dose level up to 2000 mg/kg (or above). This evidence could be considered as one element of the WoE to address acute toxicity,

2. There is **mortality or signs of severe toxicity**, leading to interim kills of the test animals, in DRF1 at 2000 mg/kg bw. Therefore the $LD50_{oral}$ of the substance is most probably below 2000 mg/kg bw and the substance does not fit in the scope of this adaptation.

A DRF2 (typically using 5 male and 5 female animals per dose and an administration period of 7 days) can also be used as a valuable element of the WoE approach, if the highest dose is 1000 mg/kg bw or higher, and if no mortality or signs of severe toxicity leading to interim humane kills of test animals for humane reasons are observed. No data are available to confirm a relation between an acute $LD50_{oral}$ > 2000 mg/kg bw and a $NOAEL_{oral}$ ≥1000 mg/kg bw, obtained after only 7 days of administration. Therefore a DRF2 as described above can only be used as one element of evidence in the WoE approach.

In summary, the DRF studies, in particular DRF1 will provide very valuable element(s) of evidence for the WoE approach. Furthermore, there would be no or only limited cost implications, as both DRF1 and DRF2 are usually performed ahead of the 28-day study.

The enhanced DRF1 should be reported as a separate study record under the acute oral toxicity section 7.2.1 in IUCLID.

3. Physico chemical data

Certain physico-chemical properties are regarded as indicative for low bioavailability and low toxicity. However, it is noteworthy that these parameters cannot be used as

standalone evidence to justify the adaptation of a systemic toxicity test, including the acute oral toxicity study. Therefore, whenever physico-chemical data are provided for the purpose of an adaptation they have to be accompanied by additional types of evidence, for example by a sub-acute oral toxicity study with a NOAEL equal to, or greater than, 1000 mg/kg bw, as specified below.

3.1. Low reactivity

Low reactivity, chemical and biological inertness or very low solubility are examples of physico chemical properties of the substances, which usually suggest that the bioavailability of the substance will be low. In the REACH registrations, relevant data on low bioavailability have been provided for some substances, which have e.g. a crystalline structure and extremely low solubility even in aggressive media (hydrogen chloride solution mimicking the gastro intestinal tract). In order to contribute to the weight of evidence, this type of data would normally need to be given as results of bioaccessibility or bioelution tests. Simulated gastric fluid and other relevant biological media need to be used in these tests to be convincing. While the bioelution method has not been accepted as an OECD Test Guideline, there is a standard protocol available as ASTM (American Society for Testing and Materials) D-5517[14] (US EPA 2008), and BARGE (Bioaccessibility Research Group of Europe). By the initiative of Eurometaux, test guideline development is ongoing aiming at an OECD test guideline project. In some read-across and trend analysis cases, bioelution studies have been found useful under REACH.

The rationale of "unreactivity" and lack of bioavailability as indicators of low toxicity is referred to in the column 2 adaptation in the Annex IX, 8.6.2. fourth indent, according to which *"the sub-chronic toxicity study (90 days) does not need to be conducted if: … the substance is unreactive, insoluble and not inhalable and there is no evidence of absorption and no evidence of toxicity in a 28-day 'limit test', particularly if such a pattern is coupled with limited human exposure."*

Non-reactive substances with very high molecular weight may also have a low bioavailability via the relevant routes of exposure. However, a high molecular weight alone is not considered to be useful data in the Weight of Evidence approach addressed in this part of the Guidance. As for any property of a substance, it also has to be considered that metabolism may influence reactivity.

If the registrant uses physico-chemical data as an element of a WoE adaptation, reliable and good quality data have to be provided with a justification of how and why a given physico-chemical property is supportive of low toxicity.

4. (Q)SAR

The use and restrictions of using (Q)SAR in order to provide information for the acute oral toxicity have been explained in Section R.7.4.3.1. Some physico-chemical parameters have been given as possible predictors of acute toxicity and it may be possible to generate relevant information with (Q)SAR methodologies, e.g. on systemically acting volatile compounds causing narcosis (Weed, 2005, Veith *et al.,* 2009) Furthermore since other methodologies (in particular NRU cytotoxicity test described below in section 4) are not appropriate for the identification of substances with specific toxic mechanisms, a QSAR modelling should be applied to find if structurally related substances have a specific mechanism. If there are indications that a substance may have a neurotoxic mechanism of action, a QSAR modelling should be applied to find if structurally related substances are neurotoxic. This indication could be based on structural similarity with a known neurotoxicant (supported by adequate read-across justification) or on mechanistic *in vivo* or *in vitro* studies. If that is the case, the

14 ASTM D-5517: extractability of metals from art materials (gastric fluid)

substance would not fit under this WoE adaptation, since neurotoxic substances often have a high acute toxicity.

Within the adaptation possibility considered in this Appendix, the core question concerning the use of (Q)SARs is whether the substance to be registered under REACH fits in the domain of a well-documented (Q)SAR model, including an open training set. If that is the case, the (Q)SAR modelling is a potential element within the WoE approach.

ECHA's Practical Guide 5 (How to report (Q)SARs)[15], illustrates the general aspects to take into account when using (Q)SAR models for regulatory purposes. It is important to distinguish between the proposed validity of the (Q)SAR model *per se*, the reliability and adequacy of an individual (Q)SAR estimate (i.e., the application of the (Q)SAR model to a specific substance), and the appropriateness of the documentation associated with models and their predictions. The appropriate documentation consists normally in a QSAR Model Reporting Format (QMRF), which documents transparently that the model is scientifically valid, and a QSAR Prediction Reporting Format (QPRF), which justifies that the prediction generated with a model for a specific substance is reliable and appropriate. Guidance on how to characterise (Q)SARs according to the OECD (Q)SAR validation principles is provided in the OECD GD 69 (OECD, 2007a).

The decision on whether to accept a (Q)SAR prediction is to be taken on a case-by-case basis.

(Q)SAR predictions may be gathered from databases (in which the predictions have already been generated and documented) or generated *de novo* through the available models. Data obtained by grouping approaches can also be used to generate local QSARs and derive a predicted toxicity value.

Programs such as the QSAR Toolbox[16] serve this purpose. This software can be used to find analogue substances that have toxicological profile similar to the substance with a data gap, which can be filled with a prediction of the relevant endpoint generated via read across or trend analysis. Furthermore, certain structures indicative of higher acute toxicity can be identified thanks to the Toolbox profilers[17].

Within this WoE adaption, it is not anticipated that QSAR prediction alone could be used to meet the information requirement. WoE by default has to consist of more than one "data element". Therefore, QSAR modelling may be useful e.g. in case it supports or confirms the evidence of low toxicity that has been obtained e.g. from the sub-acute study or from a Dose Range Finding study (see below).

5. *In vitro* cytotoxicity assay (Neutral Red Uptake)

5.1 Introduction

ECHA can only accept in vitro studies from validated and accepted methodologies. At the time of drafting of this Appendix, only the Neutral Red Uptake (NRU) test can be accepted as part of the proposed WoE approach.

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[15] http://echa.europa.eu/documents/10162/13655/pg_report_qsars_en.pdf

[16] www.qsartoolbox.org

[17] For instance, quinones are known to be able to form covalent binding with proteins via a Michael addition reaction. Aliphatic secondary amines are associated with enhanced toxicity. Pyrethroids are known to cause neurotoxicity, and therefore an increased toxicity can be expected.

The NRU *in vitro* basal cytotoxicity assay is based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye (Borenfreund and Puerner, 1985). NR is a weak cationic dye that readily diffuses through the plasma membrane and concentrates in lysosomes where it electrostatically binds to the anionic lysosomal matrix. Toxicants can alter the cell surface or the lysosomal membrane to cause lysosomal fragility and other adverse changes that gradually become irreversible. Such adverse changes cause cell death and/or inhibition of cell growth, which then decrease the amount of NR retained by the culture. Since the concentration of NR dye desorbed from the cultured cells is directly proportional to the number of living cells, cytotoxicity is expressed as a concentration-dependent reduction of the uptake of NR after chemical exposure. The amount of NR in the cells (fibroblast cell line, BALB/c 3T3) is measured with a spectrophotometer.

Based on the EURL ECVAM validation study to assess the predictive capacity of the 3T3 NRU *in vitro* cytotoxicity test to identify substances not requiring classification for acute oral toxicity (Prieto *et al.*, 2013), ECVAM has issued recommendations concerning the validity and limitations of this *in vitro* test (EURL ECVAM, 2013). Considering the results of that validation study the 3T3 NRU test method shows a high sensitivity (ca 95%) and, consequently, a low rate (ca 5%) of false negative results, when employed in conjunction with a prediction model to distinguish potentially toxic versus non-toxic (i.e. classified versus non-classified) substances. Therefore, substances found to be negative in this test would most likely not require classification for acute oral toxicity based on a cut-off value of >2000 mg / kg bw.

The validated 3T3 NRU test method appears to be particularly relevant for the assessment of industrial chemicals since they are not designed to act on specific biological targets and, in general, tend not to be acutely very toxic. Following the provisions of the REACH Regulation and in particular its Annex XI, data from the 3T3 NRU test method could be used within a WoE approach to adapt the standard information requirements.

5.2. Limitations

The 3T3 NRU test method is sensitive to hazardous chemicals acting through general mechanisms of toxicity common to most cell types, often referred to as 'basal cytotoxicity'. Consequently, chemicals, not exhibiting significant cytotoxicity but, which act through (i) **mechanisms specific only to certain cell types and tissues** (e.g. of the heart or central nervous system) may not be identified as potentially acutely toxic by this method; (ii) **metabolic activation** to induce toxicity, may go undetected since the cell model lacks significant metabolic capacity. Care must be taken therefore in interpreting negative results derived from this assay.

The 3T3 NRU test method has a high false positive rate therefore positive results cannot be readily used in a meaningful way in characterising the acutely toxic substances (i.e. acute toxicity classifications Cat 1 – Cat 4). A likely reason is that the test method does not capture important biokinetic processes such as absorption, distribution, metabolism and excretion. Thus, certain chemicals, despite having cytotoxic potential, may not actually be acutely toxic via the oral route.

Considering these limitations, results derived from the 3T3 NRU test method should **always** be **used in combination with other information** (preferably with the data from the sub-acute study) sources to build confidence in the decision not to classify a substance for acute oral toxicity. Possible complementary information sources include sub-acute toxicity study, physico-chemical properties, structural alerts, and structure–activity relationships. The *in vitro* 3T3 NRU method therefore fits within a Weight of Evidence (WoE) approach or as a component of an Integrated Testing Strategy (ITS).

Even in case the information resulting from the NRU test and QSAR models would be available, the WoE would only be accepted by ECHA if the sub-acute oral study is submitted (Table 2) and fits within the scope (i.e. NOAEL > 1000 mg/kg bw), as classification requirements must fulfilled.

5.3. Regulatory use of the *in vitro* NRU cytotoxicity test within the WoE approach

Respecting the provisions of Directive 2010/63/EU on the protection of animals used for scientific purposes, and the provisions of the REACH Regulation Article 13 and Annex XI, 1.2, the 3T3 NRU test method should be used in combination of other data, in particular the results of the sub-acute oral toxicity test. Due to its limitations the NRU test should primarily be used to correctly identify and classify the substances of low toxicity. The 3T3 NRU test method may be a valuable component of a WoE approach for supporting hazard identification and safety assessment in agreement with the EU CLP Regulation implementing the upper threshold of UN GHS Category 4 as the cut-off for non-classification of substances.

In case the sub-acute oral toxicity study has shown low toxicity of the test substance, i.e. the NOAEL of the study was above 1000 mg/kg bw, a prediction can be made that the acute toxicity is above 2000 mg/kg (see Chapter 1). In this case, the NRU test can be used to add confidence to the prediction. It should be noted that the NRU cannot be used for regulatory purposes as a stand-alone test.

The NRU test is not considered to be the only confirmatory element in the WoE approach that is primarily based on the results of the sub-acute oral test or its DRF studies. Sub-acute oral toxicity studies have higher biological relevance and better predictivity than the NRU Assay. Therefore, while the NRU test is seen as a useful element of the WoE approach, it is not regarded as an obligatory element of it. Also other types of information such as convincing data on the bioavailability or from well documented (Q)SAR modelling may be used to build confidence on the prediction, as explained elsewhere in this part of the Guidance (Chapters 3 and 4 above).

6. Other information

The WoE elements described above are the most relevant ones for the purpose of adaptations of the acute oral toxicity study. They should normally be considered, when data is collected and generated. Besides information on mechanistic and/or tissue-based *in vitro* studies (e.g. addressing neurotoxicity), there are also other useful information, which are outlined below.

6.1. Read across

The basic prerequisite to justify a read-across approach is that the source and target substances of the read-across are chemically and structurally similar, and therefore they are expected to exhibit similar properties. The target substance should not have any such functional or chemical difference, which potentially makes its properties or reactivity and its toxicity different from that of the source substance. Also a mechanistic hypothesis has to be formulated in case a registrant proposes to use a read-across argumentation. For example, very low bioavailability or lack of reactivity associated with low toxicity, or dissociation/hydrolysis to normal constituents of biological media, are hypotheses that may be associated with the read-across in support of low acute toxicity. In order to be relevant in the regulatory context, the mechanistic hypothesis needs to be supported by reliable data. Furthermore, low toxicity and low biological activity of both source and target substance of the read-across, observed in toxicity studies can be used to build confidence on the read-across justification.

Furthermore, the data that are available on the source substance and target substance must enable the prediction of the acute toxicity potential or rather the lack of it. Within the present WoE adaptation, the registrant must be able to predict, with sufficient certainty and confidence that the LD50 of the target substance of the read-across will be above 2000 mg/kg bw. While the paragraph above illustrates some principles of the read-across when applied for the purpose of this specific WoE adaptation, more detailed Guidance for the read-across can be found in R.6. and in illustrative examples given in ECHA's web-site in http://echa.europa.eu/support/grouping-of-substances-and-read-across.

The conclusions about the likely properties of a substance can also be based on the knowledge of the properties of one or more similar chemicals, by applying grouping methods. The corresponding OECD guidance provides information on the use of grouping of chemicals and read-across approaches (OECD, 2014).

6.2. Existing human data

The strength of the epidemiological evidence for specific health effects depends, among other things, on the type of analyses and on the magnitude and specificity of the response. Relevant human data may be available e.g. in reports of the poison control centres or from published case studies. Confidence in the findings is increased when comparable results are obtained in several independent studies on populations exposed to the same agent under different conditions. Other characteristics that support a causal association are the presence of a dose-response association, a consistent relationship in time and (biological) plausibility, i.e., aspects covered by epidemiological criteria such as those of Hill (1965).

A comprehensive guidance of both the evaluation and use of epidemiological and human evidence for risk assessment purposes is provided by Kryzanowski *et al.* (WHO, 2000).

High quality human data may also be obtained from historical data from individual clinics or collated clinic data and/or from dose response studies (References). High quality human data may be considered as a strong basis for C&L decision making (subject to the ethical considerations relevant for the respective regulatory programme). It is acknowledged that novel human studies are not allowed for the purpose of CLP and REACH, but existing data may be used.

The usefulness of human data in the context of this WoE adaptation is limited, since the scope of this adaptation is limited to substances of low toxicity, whereas the most definitive human data are usually available on substances which are toxic.

7. Weight-of-Evidence analysis

When applying the WoE approach, proposed in this Appendix, the registrant should aim at obtaining adequate and reliable data for hazard identification and classification purposes for the substances of low acute toxicity. Within the WoE approach, different types of data can be obtained and assessed, in order to find out whether the information requirement for the acute oral toxicity can be met, or whether further information needs to be generated.

The objective of this WoE approach is to correctly identify substances that are not acutely toxic i.e. with an $LD50_{acute,oral}$ higher than 2000 mg/kg bw and, therefore, do not need to be classified under the European CLP regulation.

7.1. Introduction

The term weight of evidence (WoE) is widely used in scientific publications and government agency guidelines in the context of risk assessment. The term has been

used in reference to a specific body of evidence without reference to interpretative method, but also methodologically, with prescribed methods addressing specific purposes such as confidence in causation (Weed, 2005).

A WoE determination means that all available and scientifically justified information bearing on the determination of hazard are considered together. In case of the acute oral toxicity, this includes animal data on sub-acute oral toxicity (including DRF studies), physico-chemical parameters, information from category approaches (e.g., grouping, read-across), (Q)SAR results, the results of suitable *in vitro* tests (e.g. validated NRU assay), and possibly human data. The quality and consistency of the data should be taken into account when weighing each piece of available information. In this context, the highest weight should be given to the sub-acute oral toxicity study and its related DRF studies, as provided for in Table 2.

A WoE approach involves an assessment of the relative values/weights of different pieces of the available information that has been gathered and generated. These weights/values can be assigned either in a more structured (even quantitative) way by applying a formalised procedure (e.g., based on Bayesian logic, as in Rorije *et al.*, 2013) or by using expert judgement. The weight given to the available evidence will be influenced by the quality of the data, consistency of results/data, and relevance of the information for the given regulatory endpoint. A matrix for the Weight of Evidence analysis is provided below (Table 2).

Examples of tools available to evaluate the quality of data include the Klimisch scores (Klimisch *et al.*, 1997) and Hill's criteria for evaluation of epidemiological data (Hill, 1965), as well as the JRC's ToxRTool for scoring *in vivo* and *in vitro* data (Schneider *et al.*, 2009). The ToxRTool[18] provides an assessment system which allows the evaluator of a given study to derive an appropriate Klimisch score.

Under the CLP Regulation Article 9(3), a WoE approach should be used when the specific criteria cannot be directly applied. According to that provision, all available information that can contribute to the determination of classification for an endpoint are considered together.

7.2. Place / role of WoE in the Assessment of acute toxicity

After the necessary testing has been performed and non-testing data have been generated and assessed, the WoE approach is applied in order to consider whether the hazard characterisation and the classification can be achieved without performing the legally required acute oral toxicity test.

As explained above and described in Table 2, the most relevant *in vivo* test is the sub-acute oral toxicity test (OECD TG 407 or 422 screening test), and the enhanced DRF1, whereas the most useful *in vitro* test is the NRU cytotoxicity test.

However, in case other relevant and good quality data can be obtained e.g. from open literature and/or from the own data bases of the registrant, a WoE analysis could actually be performed, but not necessarily completed, even before performing new *in vitro* or *in vivo* tests. In case a WoE analysis is based on available data, there are two possible conclusions, either the data is considered sufficient and a WoE adaptation is submitted in the registration dossier without novel testing, or the WoE based on the available data remains insufficient or inconclusive and generation of further data is necessary.

[18] https://eurl-ecvam.jrc.ec.europa.eu/about-ecvam/.../**toxrtool**/**ToxRTool**.xls

Considering human evidence, several types of existing information can be used, provided these are of sufficient quality. In the WoE analysis, the availability of the specified types of data should be checked. The sources of those data may vary, ranging from clinical study reports, scientific publications, data from poison information centres, guideline tests, to worker surveillance data from the chemical industry.

7.4. Assessment of data quality

The quality of the data obtained for a WoE approach needs to be assessed, since the quality will contribute to the weight of each data element. In case the quality of a certain study is deemed to be inappropriate, those data should not be included in the WoE. Instead it is recommended to focus on other elements of information, which are of sufficient quality. Quality might be inappropriate e.g., due to the missing validation of a methodology, the "non-adherence" to the relevant test guideline/method, the lack of adequate controls, and/or the deficiencies in data reporting, etc.

The quality of toxicological studies is usually described by assigning Klimisch scores. Epidemiological data can be evaluated using Hill's criteria (Hill, 1965).

For many existing chemicals, it is acknowledged that some of the available information may have been generated prior to the requirements of Good Laboratory Practice (GLP) and/or prior to the acceptance of the standardised OECD test methods. While such information may still be usable, both the data and the methodology used must be evaluated in order to determine their reliability. Such an evaluation would ideally require an evidence-based evaluation i.e., a systematic and consistent evaluation following pre-defined, transparent and independently reviewed criteria before making decisions. These should always include justifications for the use of particular data sets on the basis of the criteria-based evaluation.

7.5. Adequacy and relevance of information

The "Adequacy" of information defines the usefulness of information for the purpose of hazard and risk assessment, i.e. whether the available information contributes to the decision-making on whether the chemical is acutely toxic, and whether an adequate classification can be derived. The evaluation of adequacy of test results, and documentation for the intended purpose, are particularly important for chemicals, where a number of test results are available, but where some (or all) of them have not been carried out according to current standards. Where there is more than one study, the greatest weight is given to the studies that are the most relevant and reliable (e.g. validated and/or regulatorily approved).

7.6. Evaluation of consistency of the data

The consistency of the existing data from various sources is crucial and should therefore be thoroughly evaluated during the WoE approach. In case the elements of evidence are of comparable weight but give inconsistent evidence, usually the WoE analysis will not be conclusive enough. Consequently *in vivo* and/or *in vitro* testing will have to be considered and conducted. In case the weights of the individual pieces of evidence differ considerably (e.g., inconsistent results obtained from *in vitro* and/or *in vivo* testing and human data), a WoE conclusion may be drawn according to the evidence carrying the highest weight. It is important to evaluate what the reasons for inconsistent data e.g. from *in vitro* methods may be, and whether the lack of metabolic capacity affects the prediction. In case the inconsistency cannot be scientifically explained, the WoE analysis becomes inconclusive, and therefore the WoE-based adaptation should not be proposed by the registrant. Consistent data, on the other hand, which come from several studies

and/or sources may be considered sufficient for regulatory purposes, pursuant to Annex XI, section 1.2.

7.7. Conclusions from the WoE analysis

The core and minimum element of the WoE approach proposed in this Appendix is the sub-acute oral toxicity study performed with the registered substance. Where properly justified and documented, a sub-acute oral toxicity study with a read-across substance may be proposed, according to Annex XI, section 1.2. In addition, one or more other WoE elements are needed. Furthermore, the registrants need to justify, why their combination of the WoE elements is sufficient and how they have minimised the uncertainty associated with the WoE approach.

In the final analysis of the WoE approach, each element of evidence must be characterised for its quality, relevance, coverage and consistency with other information (see the “Matrix for the Weight of Evidence analysis”, Table 2).

When consistency is seen among ”qualified” element of evidence, the WoE analysis may reach a conclusion that the relevant information requirement has been sufficiently covered, and that further *in vivo* testing is not necessary. In that case, a conclusion can also be drawn that the substance does not need to be classified for acute toxicity (Figure 2).

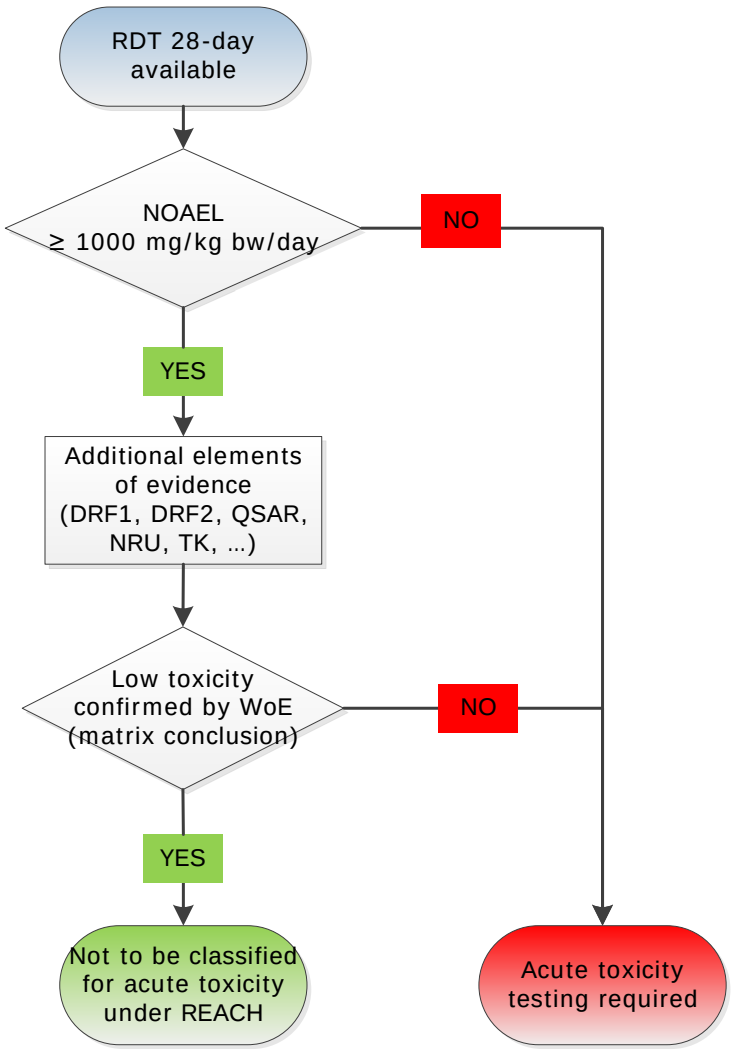

Figure 2: Decision tree to assess whether an *in vivo* acute toxicity test is required, when the registrant holds an **existing repeated sub-acute oral toxicity study**, and makes use of the WoE approach.

In case the existing study was performed on an analogue substance, it is the registrants' responsibility to justify the read-across approach proposed. Where ECHA would accept the justification, the study could be used as part of the WoE analysis.

When, on the other hand, insufficient information remains after the "non-qualified" data have been rejected and/or when the remaining information is inconsistent or contradictory, the WoE analysis would reach the conclusion that the relevant endpoint, or information requirement, has not been sufficiently covered and, that further *in vivo* testing is necessary, according to the specific legal/regulatory framework.

The WoE justification has to be specific for the registered substance and specific to the set of data information used by the registrant in order to meet the corresponding information requirement.

After collecting and assessing the data, the registrants need to decide how to include the existing information in the registration data set. It is recommended that each element of evidence of the WoE is included in the registration dossier as an individual study record, in IUCLID Section 7.2. Furthermore, the WoE analysis and its conclusion may be

included in the summary of the Section 7.2. The matrix given below can be used for preparing that summary.

8. In vivo acute toxicity test

Due to the limitations of the methods and types of information described above, there are cases where the acute oral toxicity study will be needed, e.g.

- based on the DRF or on the results of the sub-acute toxicity study, the $LD50_{acute,oral}$ is lower or is likely to be lower or equal to the limit of 2000 mg/kg (C&L limit) and, therefore, the substance does not fall in the domain of this WoE adaptation,
- the information obtained and results of the tests performed are inconsistent and this inconsistency cannot be scientifically explained,
- the registrant has to conclude on classification of acute toxicity category 5, i.e. $LD50_{acute,oral}$ is comprised between 2000 mg/kg bw and 5000 mg/kg bw, e.g. because the substance is placed on the market in a country where the authority has implemented that category, and/or
- the registrant may have some existing (e.g. structural data) information that the substance may be acutely toxic and the registrant aims to ensure the proper level of risk management measures.

In these cases, the registrant is advised to document, why data brought to the WoE analysis were not sufficient in fulfilling this information requirement, and consequently a relevant test according to the OECD/EU guidelines is needed (according to Article 13).

Table 2. **Matrix for the Weight of Evidence analysis**.

Fill in the entries for those modules, for which data is available or generated. It is recommended that the results of a sub-acute study are always included in the WoE analysis. In addition, one or more other elements of evidence need to be provided. The type of other available information or which can be generated, will vary depending on the case. For any remaining entries, indicate NA (not available) in the respective column.

Module	**Title of document/full reference or data not available (NA)**	**Study result, evidence obtained**	**Data quality,** according to the Klimisch score, when appropriate	**Adequacy and relevance,** short statement	**Coverage of relevant parameters and observations, *3)***	**Consistency with other information, *1)***	**Conclusive remark, *2)***
1. Sub- acute toxicity study				Highest relevance			
2. Enhanced DRF1				High relevance (usually)			
3.*In vitro* cytotoxicity assay (NRU)				Only negative results are relevant			
4. (Q)SAR modelling	*i.e. QMRF*	*i.e. Predicted value*		Relevant if applicability domain is considered appropriate			
5. Physico-chemical properties				Relevant when available			
6. Other data (existing human data, read-across				Case-by-case			
Overall conclusion	1. WoE allows assessment of acute toxicity of the substance. The substance should be classified as not acutely toxic, **or** 2. WoE does not allow assessment of acute toxicity of the substance. The registrant needs to consider the most appropriate additional testing, which would usually be an acute toxicity test performed according to a relevant OECD test guideline.						

1) For example: *"This element of evidence (any entry except 1 and 2) is consistent with the sub-acute toxicity study".*

2) For example: *"The existing human data suggest that the substance is not acutely toxic. Due to poor reporting of this data, and low quality in terms of exposure information, the data is inconclusive, and has a low weight in the final evaluation.* "

3) Definition of the relevant parameters for each element of the WoE, when applicable.

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Appendix R.7.4-2 (Q)SARs for the prediction of acute toxicity

Below some examples are given in order to illustrate the prospects for applying the (Q)SAR approaches for the acute toxicity endpoint for predictive purposes or to investigate the mechanisms of toxicity.

(Q)SARs on inhalation toxicity

Some simple regression models have been developed for predicting the inhalational toxicity of volatile substances, and these can be used reliably within their domains of applicability. Typically, parameters such as vapour pressure (VP) and boiling point (BP) have been found to be useful predictors of the acute toxic effect (e.g. LC_{50} value). These models are based on the assumption that toxicity occurs by the non-specific mechanism of narcosis, and that the LC_{50} data are based on tests in which a steady-state concentration has been reached in the blood. These models are suitable only for systemic acting volatile compounds.

For example, acute (non-lethal) neurotoxicity data for the neurotropic effects of some common solvents on both rats (whole-body exposures for 4h) and mice (whole-body exposures for 2h), taken from Frantik *et al.* (1994), were subjected to QSAR analysis by Cronin (1996). Stepwise regression analysis of the 4-hr toxicity data causing the 30% depression in response ($log1/ECR_{30}$) in rats gave the following equation:

$$log1/ECR_{30} = 0.361\ ClogP - 0.117\ {}^{0}\chi - 1.76$$

$$n = 37 \qquad R^2 = 0.817 \qquad s = 0.280 \qquad F = 35.2$$

This relationship demonstrates a partial dependence of neurotoxicity with the octanol-water partition coefficient, logP. The negative correlation with the zero-order molecular connectivity ${}^{0}\chi$ is thought to be an indication that the membrane permeability of blood-brain barrier is reduced for large molecules.

Stepwise regression for mouse neurotoxicity gave the following equation:

$$log1/ECM_{30} = 0.212\ ClogP + 0.00767\ BP - 0.176\ {}^{0}\chi - 2.03$$

$$n = 39\ R^2 = 0.811 \qquad s = 0.271 \qquad F = 22.4$$

in which BP is the boiling point of the substance (BP is inversely related to vapour pressure).

The application of principal components analysis (PCA), to separate compounds of high neurotoxicity from those of low neurotoxicity, suggested that in addition to partitioning through a membrane (determined by logP and molecular size), aqueous solubility and volatility are also important factors governing neurotoxicity (Cronin, 1996). Metabolism to more toxic compounds is suggested as a possible cause of compounds appearing as outliers in the QSARs.

Regarding baseline inhalation toxicity, Veith *et al.* (2009) developed two models for the prediction of narcosis in rodents using data from inhalation toxicity studies on mice and rats from the US ECOTOX database:

$$Log\ LC50_{rat} = 0.69\ logVP + 1.54$$

$$n = 36\ r^2 = 0.94 \qquad Std.\ Error = 0.19 \qquad StT\ test = 18.35$$

$$\text{Log } LC50_{mouse} = 0.57 \text{ logVP} + 2.08$$

$$n = 28\ r^2 = 0.74 \qquad \text{Std. Error} = 0.20 \qquad \text{StT test} = 8.63$$

where VP is the estimated vapour pressure of the substance using EPISUITE v3.2. in mm Hg.

The models are not suitable for reactive substances or those exerting receptor mediated toxicity. An approach taken in the development of the models was to exclude those substances identified as reactive by the Russom scheme (Russom *et al.*,1997).

QSARs for predicting LD_{50}

There are references in the literature to a few models for predicting LD_{50}, generally for small sets of compounds. For example, Hansch and Kurup (2003) developed the following QSAR to predict the toxicity of barbiturates (LD_{50}) in for female white mice, using toxicity data from Cope and Hancock (1939):

$$\log 1/LD_{50} = -1.44 \log P + 0.16\ NVE - 8.70$$

$$n = 11\ R^2 = 0.924 \quad s = 0.077 \quad R^2_{cv} = 0.879$$

where NVE is the number of valence electrons (used as a measure of polarisability).

More recently, Koleva *et al.* (2011) developed two nonlinear regression models to quantify the oral LD50 for compounds causing only baseline toxicity in rats and mice:

$$\text{log } 1/LD50 \text{ rat} = -1.780 + 0.465 \text{ logP} - 0.111(\text{logP})^2$$

$$n = 55 \qquad \text{rms} = 0.15 \quad r^2_{adj} = 0.59 \quad F = 40.3$$

$$\text{log } 1/LD50 \text{ mouse} = -1.841 + 0.503 \text{ logP} - 0.105(\text{logP})^2$$

$$n = 30 \qquad \text{rms} = 0.17 \quad r^2_{adj} = 0.72 \quad F = 38.5$$

were logP is the n-octanol/water partition coefficient.

The models were developed with a training set of saturated monohydric alcohols and saturated monoketones. Substances with limited water solubility or potentially undergoing metabolism were considered out of the domain, and excluded from both training and test sets. The authors highlight some classes of reactive substances that are out of the domain since they exert excess toxicity, particularly electrophilic substances that are able to undergo covalent binding to nucleophilic sites.

QSARs for predicting human toxicity

The same descriptors were used to predict the LD_{100} of miscellaneous drugs to humans, using toxicity data from King (1985):

$$\log 1/C = 0.61 \log P + 0.017\ NVE + 1.44$$

$$n = 36 \qquad R^2 = 0.850 \quad s = 0.438 \quad R^2cv = 0.817$$

QSARs for predicting *in vitro* effects

A number of QSAR models for predicting *in vitro* effects are cited in the literature (reviewed in Tsakovska *et al.*, 2006), but these are not directly relevant to the assessment of acute toxicity for regulatory purposes. In general, these models have been developed to investigate the mechanisms of cytotoxic action, and they outline the role of hydrophobicity as well electronic descriptors, including electrotopological state descriptors (Lessigiarska *et al.*, 2006), bond dissociation energies (Selassie *et al.*, 1999), and dissociation constants (Moridani *et al.*, 2003). While these models are not directly relevant to the assessment of acute toxicity, the fact that reliable QSARs can be developed for the *in vitro* cytotoxicity of defined groups of substances indicates that the approach of modelling *in vitro* data should be further explored with a view to integrating such QSARs into the ITS for acute toxicity. For example, a battery of QSARs could be developed for predicting the *in vitro* data of a validated *in vitro* test, and then used to supplement or replace *in vivo* testing.

Computerised models

For heterogeneous groups of compounds, computerised models are available to predict acute toxicity (normally $LD50_{oral}$).

Knowledge-based software (see also Section R.6.1 of Chapter R.6 of the *Guidance on IR&CSA*), such as HazardExpert, are based on rules derived from human expert opinion to estimate toxicity. In statistically based software, such as TOPKAT and MultiCASE, statistical methods are used to derive (Q)SAR models (see also Section R.6.1).

A list of some of the available computerised models with a brief description is provided below.

OECD QSAR Toolbox

The freely available for download OECD QSAR Toolbox software (http://www.qsartoolbox.org/) contains profilers that could be useful in creating mechanistic categories for acute oral toxicity in rats:

- Toxic hazard classification by Cramer, which assigns the substance to a toxicity class (“High”,”Medium” or “Low”) based on the effects when administered orally.
- Protein binding by OASIS and Protein binding by OECD, which allows identifying electrophilic substances, which are likely to exhibit higher acute toxicity due to their reactivity.
- Repeated dose toxicity (HESS), which was initially developed by the Japanese NITE with a view to help predicting effects in a 28 days study in rats. The profiler would allow to identify some specific modes of action that are also relevant for acute toxicity (e.g. neurotoxicity).

The QSAR Toolbox also contains experimental data on acute toxicity in the following databases:

- ECHA Chem: this database contains non-confidential data from REACH registration dossiers

- Rodent Inhalation Toxicity Database: it is a compilation of high quality data from rat inhalation studies reported in the literature.

- Toxicity Japan MHLW: it contains experimental results from single dose toxicity test and mutagenicity test results performed under Japan's Existing Chemicals Programme.

The use in combination of profilers and data for analogues could allow the prediction of acute oral toxicity for new substances through a read-across or trend analysis approach.

HazardExpert

HazardExpert is a module of Pallas software developed by CompuDrug Limited (http://www.compudrug.com). The program works by searching the query structure for known toxicophores, which are stored in the "Toxic Fragments Knowledge Base" and which include substructures exerting both positive and negative modulator effects. Once a toxicophore has been identified, this triggers estimates for a number of toxicity endpoints, including neurotoxicity. The default knowledge base of the system is based on a US-EPA report (Brink and Walker, 1987) and scientific information collected by CompuDrug Limited. This program can be linked to MetabolExpert, another module of the Pallas software, to predict the toxicity of the parent compound and its metabolites. Information on the validity of the model is not available. Investigations on the validity and applicability of HazardExpert are needed before recommendations can be made about its regulatory use.

TOPKAT

The TOPKAT software package employs cross-validated quantitative structure-toxicity relationship (QSTR) models for assessing various measures of toxicity (http://accelrys.com/products/collaborative-science/biovia-discovery-studio/qsar-admet-and-predictive-toxicology.html). The Rat Oral LD_{50} module of TOPKAT includes 19 QSAR regression models for different chemical classes. The models are based on a number of structural, topological and electrophysiological indices, and they make predictions of the oral acute median lethal dose in the rat (LD_{50}).

The TOPKAT rat oral LD_{50} models are based on experimental data from the Registry of Toxic Effects of Chemical Substances (RTECS). Since RTECS lists the most toxic value when multiple values exist, the TOPKAT model tends to overestimate the toxicity of query structures.

The Rat Inhalation LC_{50} module of TOPKAT contains five submodels related to different chemical classes.

TOPKAT models, including the models for acute oral toxicity, were used by Danish EPA (http://qsar.food.dtu.dk/) in 2005 to evaluate the dangerous properties of around 47,000 organic substances on the EINECS list. An external evaluation of this model using 1840 substances not contained in the TOPKAT database gave poor results (R^2 = 0.31). However, 86% of estimations fall within a factor of 10 from test results (DK EPA study).

The Danish EPA concluded that the TOPKAT model is sufficient to give an indication of the least strict classification for acute toxicity, Xn; R22.

CASE Ultra

CASE Ultra software (http://www.multicase.com) contains an acute toxicity module, which consists of a rat LD_{50} model based on 12,262 compounds from compilations by NTP, WHO, RTECS, and other regulatory agencies data. Information on the validity of the

model is not available. Investigations on the validity and applicability of CASE Ultra are needed before recommendations can be made about its regulatory use.

T.E.S.T.

The Toxicity Estimation Software Tool (T.E.S.T.), developed by the US EPA allows the prediction of many different endpoints, including oral LD50 in rats. Version 4.0 and greater contain a database of 7413 substances with rat acute toxicity data that can be used with different methods to build a model for the prediction of LD50, such as hierarchical clustering, random forest, use of nearest neighbors and a consensus model. The software uses a variety of molecular descriptors to perform the predictions. The accuracy of the predictions for LD50 depends on the model use and the type of the substance, but, according to the software documentation, overall it is not as good as for other endpoints.

The software allows to visualise the closest analogues in the training set and the test set of the models, and accuracy of each model for them, so that the user can use expert judgement to estimate whether a prediction is reliable.

Derek Nexus

Derek Nexus (http://www.lhasalimited.org/products/derek-nexus.htm) contains sets of structural alerts for many human health endpoints. Amongst them there are several alerts for “high acute toxicity”, which cannot be used to derive directly a LD50, but can be of use in identifying very toxic compounds. The alerts for other endpoints can be used to identify molecules with specific modes of action would would be expected to be of particular toxicity due to these effects, such as cardiotoxicity or cholinesterase inhibition.

ACD/Percepta

The models contained in the ACD/Percepta suite (http://www.acdlabs.com/products/percepta/) allow the calculation of LD50 for mouse under oral, intraperitoneal, intravenous, subcutaneous administration and for rats under oral and intraperitoneal administration methods. All of them are based on fagmental QSARs used to derive baseline toxicity, plus corrections for excess toxicity based on fragments associated with specific modes of action. More than 100 000 compounds were used in the development of the models, although it is unclear on how many data points each model was based. The software provides an automatic assessment of the reliability of the prediction based on the similarity of the compounds in the training set and the accuracy of the predictions for them.

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