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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.7.5 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 4.1 published in October 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 6.0	Full revision addressing the content of Section R.7.5 related to Repeated dose toxicity. The update includes the following: • Sections R.7.5.3.1 "Non-human data on repeated dose toxicity" and R.7.5.3.1.1 "Non-testing data on repeated dose toxicity": Text regarding OECD HPV and ECB work on QSAR models removed or updated; addition of new Appendix R.7.5-2 on relevant QSAR models; addition of cross-references to relevant practical guides; • Section R.7.5.3.1.2 "Testing data on repeated dose toxicity": Editorial changes; addition of the extended one generation reproductive toxicity test in table R.7.5-2 for other studies relevant for evaluation of existing information on repeated dose toxicity. • Section R.7.5.4.1.1 "Non-testing data on repeated dose toxicity": Updated text on read across taking into account experience from evaluation work and updated practical guides and guidance documents on the topic; • Section R.7.5.4.1.2 "Testing data on repeated dose toxicity" in the "Animal data" sub-section: text revision to update some reference guidance documents on Mode of action and Immunoxicity; some text more relevant to the ITS section was moved there; example of carcinogenicity studies were removed; • Table R.7.5-2: Update taking into account updated OECD TGs; • Section R.7.5.6.2 "Preliminary considerations": addition of text to link with Section R.7.4 on how to use sub-acute oral toxicity data for acute toxicity testing adaptations; • Section R.7.5.6.3 "Testing strategy for repeated dose toxicity": addition of a note to indicate that the latest TG update should be considered; • Section R.7.5.6.3.4 "Further considerations for studies that will be performed": section updated to put forward the route of administration selection taking into account experience from evaluation work; additional investigations section revised to have kinetics, mode of action, specific section on neurotoxicity, immunotoxicity, BAL and endocrine disruption with reference to latest guidance updates from other international bodies and to align it with ECHA Biocides Guidance on repeated dose toxicity; • References: list revised/corrected.	XXX 201X

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R.7.5 Repeated dose toxicity

R.7.5.1 Introduction

Repeated dose toxicity studies provide information on possible adverse general toxicological effects likely to arise from repeated exposure to a substance. Furthermore, these studies may provide information on e.g. reproductive toxicity and carcinogenicity, even though they are not specifically designed to investigate these endpoints.

Organs and tissues investigated in repeated dose toxicity studies include vital organs such as heart, brain, liver, kidneys, pancreas, spleen, immune system, lungs etc. Effects examined may include changes in morphology, physiology, growth or life span, behaviour which result in impairment of functional capacity or impairment of capacity to compensate for additional stress or increase in the susceptibility to the harmful effects of other environmental influences. Therefore, it is important that the possible adverse general toxicological effects are assessed for chemical substances that may be present in the environment.

R.7.5.1.1 Definition of repeated dose toxicity

The term *repeated dose toxicity* comprises the general toxicological effects occurring as a result of repeated daily dosing with, or exposure to, a substance for a part of the expected lifespan (sub-acute or sub-chronic exposure) or for the major part of the lifespan, in case of chronic exposure.

The term *general toxicological effects* (in this report often referred to as *general toxicity*) includes effects on, e.g. body weight and/or body weight gain, absolute and/or relative organ and tissue weights, alterations in clinical chemistry, urinalysis and/or haematological parameters, functional disturbances in the nervous system as well as in organs and tissues in general, and pathological alterations in organs and tissues as examined macroscopically and microscopically. Repeated dose toxicity studies may also examine parameters that have the potential to identify specific manifestations of toxicity such as e.g., neurotoxicity, immunotoxicity, endocrine-mediated effects, reproductive toxicity and carcinogenicity.

An *adverse effect* is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system, or (sub) population that results in an impairment of functional capacity, or an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences (OECD, 2003).

A chemical substance may induce systemic and/or local effects.

- A *local effect* is an effect that is observed at the site of first contact, caused irrespective of whether a substance is systemically available.

- A *systemic effect* is defined as an effect that is normally observed distant from the site of first contact, i.e. after having passed through a physiological barrier (mucous membrane of the gastro-intestinal tract or of the respiratory tract, or the skin) and becomes systemically available.

- It should be noted, however, that toxic effects on surface epithelia may reflect indirect effects as a consequence of systemic toxicity or secondary to systemic distribution of the substance or its active metabolite(s).

R.7.5.1.2 Objective of the guidance on repeated dose toxicity

The objectives of assessing repeated dose toxicity are to evaluate:

- whether exposure of humans to a substance has been associated with adverse toxicological effects occurring as a result of repeated daily exposure for a part of the expected lifetime or for the major part of the lifetime; these human studies potentially may also identify populations that have higher susceptibility;
- whether administration of a substance to experimental animals causes adverse toxicological effects as a result of repeated daily exposure for a part of the expected lifespan or for the major part of the lifespan; effects that are predictive of possible adverse human health effects;
- the target organs, potential cumulative effects and the reversibility of the adverse toxicological effects;
- the dose-response relationship and threshold for any of the adverse toxicological effects observed in the repeated dose toxicity studies;
- the basis for risk characterisation and classification and labelling (C&L) of substances for repeated dose toxicity.

R.7.5.2 Information requirements for repeated dose toxicity

Section R.2.1 in Chapter R.2 of the *Guidance on IR&CSA* provides general guidance on the information requirements of the REACH Regulation. For repeated dose toxicity, all available information relevant for the endpoint needs to be evaluated and classification under Regulation (EC) No 1272/2008 on the *Classification, labelling and packaging of substances and mixtures* (CLP Regulation) considered at each tonnage level. The following standard information requirements on repeated dose toxicity are specified in Annexes VII-X to the REACH Regulation:

- In **Annex VII** (≥ 1 t/y), no test requirements on repeated dose toxicity are specified additionally to the available information relevant for repeated dose toxicity.
- In **Annex VIII** (≥ 10 t/y), a short-term repeated dose toxicity study (28 days) is usually required, in one species, male and female, using the most appropriate route of administration, having regard to the likely route of human exposure.
- In **Annex IX** (≥ 100 t/y), a sub-chronic repeated dose toxicity study (90-days) is usually required, in one species (90-day study in rodents), male and female, and a short-term repeated dose toxicity study (28 days) is the minimum requirement, using the most appropriate route of administration, having regard to the likely route of human exposure. It should be noted that a 28-day test is not required at this tonnage level if already provided as part of Annex VIII requirements or if a 90-day study is proposed at this tonnage level.
- In **Annex X** (≥ 1000 t/y), no specific test requirements additionally to those required in Annexes VIII-IX for repeated dose toxicity are required at this tonnage level.

Column 1 of Annexes VII-X to the REACH Regulation establishes the standard information required for all chemical substances and Column 2 lists specific rules according to which the required standard information requirements for individual endpoints may be modified (adapted) by waiving the requirement(s) for certain information, or in certain cases, defining

the need for additional or different information (for further details see Section R.2.1 in Chapter R.2 of the *Guidance on IR&CSA*).

In addition to the specific rules for adaptation listed in column 2 of Annexes VII to X, the required standard information may also be adapted according to Annex XI, which specifies general rules for adaptation of the standard testing requirements set out in Annexes VII-X in cases where 1) testing does not appear scientifically necessary, 2) testing is technically not possible, and 3) testing may be omitted based on the exposure scenarios developed in the CSA (substance-tailored exposure-driven testing) (see Section R.5.1 "Exposure based waiving" in Chapter R.5 of the *Guidance on IR&CSA*).

It should also be noted that the introductory sections to Annexes VII-X point to specific adaptations of the standard information requirements as *in vivo* testing must be avoided with corrosive substances at concentration/dose levels causing corrosivity.

Factors that can influence the standard information requirements include the results of other toxicity studies, immediate disintegration of the substance, accumulation of the substance or its metabolites in certain tissues and organs, failure to identify a NOAEL in the required test at a given tonnage level, toxicity of particular concern, exposure route, structural relationships with a known toxic substance, physico-chemical properties of the substance, and use and human exposure patterns. These adaptations are detailed in the stepwise Integrated Testing Strategy (ITS) presented in Section R.7.5.6.

R.7.5.3 Information sources on repeated dose toxicity

Toxicological information, including repeated dose toxicity, can be obtained from unpublished studies, databases 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). Information relevant for repeated dose toxicity can also be obtained from data on other endpoints, structural analogues and physico-chemical properties.

Before new tests are carried out to determine the hazardous properties of a substance, all available information must be assessed, according to step 1 of Annex VI to the REACH Regulation (see Chapter R.4 of the *Guidance on IR&CSA* for general guidance on the evaluation of information).

R.7.5.3.1 Non-human data on repeated dose toxicity

R.7.5.3.1.1 Non-testing data on repeated dose toxicity

Physico-chemical data

The physico-chemical properties of a substance are essential elements to be considered when selecting a suitable vehicle for dosing of the tested substance, when deciding on the appropriate administration route to be applied in experimental *in vivo* repeated dose toxicity studies as well as when deciding on exemption from testing in cases where testing is technically not possible.

(Q)SAR models

Compared with some other endpoints, the possibility to use (Q)SAR models for the prediction of repeated dose toxicity in a regulatory context is limited. This limitation is due to the complexity of the systemic interactions and effects involved in repeated dose toxicity studies. This complexity is difficult to predict with computational tools. Therefore the use of (Q)SAR models should be seen in the context of *Weight-of-Evidence* considerations, where screening and mechanistic information (including the prediction of target organs) from (Q)SARs can

support the decision on the most appropriate testing stategy. The (mostly commercial) (Q)SAR models for repeated dose toxicity are described in Appendix R.7.5-2.

More extensive guidance on the availability and application of (Q)SARs is available in Section R.6.1 in Chapter R.6 of the *Guidance on IR&CSA* and in ECHA *Practical Guide 5* on "How to use and report (Q)SARs" available on the ECHA website.

Structurally or mechanistically related substance(s) (read-across/chemical category)

The concept of grouping, including both read-across and the related chemical category concept has been developed under the OECD HPV programme (OECD 2007a). This is an approach which might be used to fill data gaps without the need for conducting tests when specific conditions, as specified in Section 1.5 of Annex XI to the REACH Regulation, are met.

Extensive guidance on the application of chemical categories/read across is available in Section R.6.2 in Chapter R.6 of the *Guidance on IR&CSA*.

R.7.5.3.1.2 Testing data on repeated dose toxicity

In vitro data

Currently, no available alternatives to animal testing are accepted for regulatory purposes for detecting toxicity after repeated exposure. Numerous *in vitro* systems have been developed over the last decades and have been discussed and summarized in recent EURL ECVAM reports on repeated dose toxicity testing (Worth and Balls, 2002; Prieto *et al.*, 2005; Prieto *et al.*, 2006). At present, the *in vitro* models listed in these reports are at the research and development level and cannot be used for repeated dose toxicity prediction purposes, although they are very useful to study individual types of organ toxicity or to assess mechanistic aspects of target organ toxicity, at the tissue, cellular and molecular levels. Some of the limitations of these models include for instance the limited capacities of current cell culture systems to account for kinetics and biotransformation, and the difficulty to derive values such as NOAELs from *in vitro* systems. Further development and optimisation of current *in vitro* systems as well as the selection of endpoints relevant to general as well as cell-type-specific mechanisms of toxicity or expression of toxic effects *in vivo* is ongoing. New technologies such as genomics, transcriptomics, proteomics and metabolomics could help in the identification of specific markers of toxicity that occur early in the process of long-term toxic responses and that are mechanistically linked to the underlying pathology. An EURL ECVAM workshop report (Prieto *et al.*, 2006) includes a proposed approach to assess repeated dose toxicity *in vitro* by integrating physiologically-based kinetic (PBK) modelling, the use of biomarkers, and omics technologies. However, this integrated approach is still under development and evaluation and it is not ready for regulatory purposes.

The latest information on the status of alternative methods that are under development can be obtained from the EURL ECVAM website (https://eurl-ecvam.jrc.ec.europa.eu/) and other international centres for validation of alternative methods.

In vitro data using human cell lines, particularly on kinetics and metabolism, may assist in study interpretation thereby avoiding the need for unnecessary animal experimentation.

At present, available *in vitro* test data from well-characterised target organ and target system models on, e.g. mode of action(s) / mechanism(s) of toxicity may be useful in the interpretation of observed repeated dose toxicity.

Animal data

The most appropriate data on repeated dose toxicity for use in hazard characterisation and risk assessment are primarily obtained from studies in experimental animals conforming to internationally agreed test guidelines. In some cases repeated dose toxicity studies not conforming to conventional test guidelines may also provide relevant information for this endpoint.

The information that can be obtained from the available EU/OECD test guideline studies for repeated dose toxicity is briefly summarised below.

Table R.7.5–1 summarises the parameters examined in these OECD test guideline studies in more detail and gives an overview of the similarities and differences between the various studies. It should be noted that the test guidelines given in the Annex to the EU Test Methods (TM) Regulation (Council Regulation (EC) No 440/2008) are initially comparable to the OECD test guidelines (http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm). However, several OECD test guidelines for repeated dose toxicity (e.g. OECD TGs 407, 412, 413) have recently been updated with significant new information but those changes have not yet been implemented in the EU TM Regulation. Hence, for conducting new tests, the latest update of a test guideline (OECD TG and/or EU method) should be used. Further details of the study protocols are described in the respective test guidelines.

- Repeated dose 28-day toxicity studies:

Separate guidelines are available for studies using oral administration (OECD TG 407 / EU B.7), dermal application (OECD TG 410 / EU B.9), or inhalation (OECD TG 412 / EU B.8). The principle of these study protocols is identical although the OECD TG 407 protocol includes additional parameters compared to those for dermal and inhalation administration, enabling the identification of a neurotoxic potential, immunological effects or reproductive organ toxicity. In addition, OECD TG 407 allows certain endocrine mediated effects to be put into context with other toxicological effects.

The 28-day studies provide information on the toxicological effects arising from exposure to the substance during a relatively limited period of the animal's life span.

- Repeated dose 90-day toxicity studies:

Separate guidelines are available for studies using oral administration (OECD TGs 408 and 409 / EU B.26 and B.27 in rodent and non-rodent species, respectively), dermal application (OECD TG 411 / EU B.28), or inhalation (OECD TG 413 / EU B.29). The principle of these study protocols is identical although the revised OECD TG 408 protocol includes additional parameters compared to those for dermal and inhalation administration, enabling the identification of a neurotoxic potential, immunological effects or reproductive organ toxicity.

The 90-day studies provide information on the general toxicological effects arising from sub-chronic exposure (a prolonged period of the animal's life span) covering post-weaning maturation and growth well into adulthood, on target organs and on potential accumulation of the substance.

- Chronic toxicity studies:

The chronic toxicity studies (OECD TG 452 / EU B.30) provide information on the toxicological effects arising from repeated exposure over a prolonged period of time covering the major part of the animal's life span. The duration of the chronic toxicity studies should be at least 12 months.

The combined chronic toxicity / carcinogenicity studies (OECD TG 453 / EU B.33) include an additional high-dose satellite group for evaluation of pathology other than neoplasia. The satellite group should be exposed for at least 12 months and the animals in the carcinogenicity part of the study should be retained in the study for the majority of the normal life span of the animals.

Ideally, the chronic studies should allow for the detection of general toxicity effects (physiological, biochemical and haematological effects, etc.) but could also inform on neurotoxic, immunotoxic, reproductive and carcinogenic effects of the substance. However, in 12-month studies, non-specific life shortening effects, which require a long latent period or are cumulative, may possibly not be detected in this study type. In addition, the combined study will allow for detection of neoplastic effects and a determination of a carcinogenic potential and the life-shortening effects.

- The combined repeated dose toxicity study with the reproduction/developmental toxicity screening test:

The combined repeated dose toxicity / reproductive screening study (OECD TG 422[1]) provides information on the toxicological effects arising from repeated exposure (generally oral exposure) over a period of minimum 4 weeks for males and approximately 63 days for females (a relatively limited period of the animal's life span) as well as on reproductive toxicity. For the repeated dose toxicity part, OECD TG 422 is in concordance with OECD TG 407 / EU B.7 except for the use of pregnant females, for which exposure duration (of female animals) is longer in OECD TG 422 compared to OECD TG 407 / EU B.7.

- Neurotoxicity studies:

The neurotoxicity study in rodents (OECD TG 424 / EU B.43) has been designed to further characterise potential neurotoxicity observed in repeated dose systemic toxicity studies. The neurotoxicity study in rodents will provide detailed information on major neuro-behavioural and neuro-pathological effects in adult rodents.

- Delayed neurotoxicity studies of organophosphorus substances:

The delayed neurotoxicity study (OECD TG 419 / EU B.38) is specifically designed to be used in the assessment and evaluation of the neurotoxic effects of organophosphorus substances. This study provides information on the delayed neurotoxicity arising from repeated exposure over a relatively limited period of the animal's life span.

[1] To date there is no corresponding EU test method available.

1 **Table R.7.5–1 Overview of *in vivo* repeated dose toxicity test guidelines**

Test	Design	Endpoints
OECD TG 407 (EU B.7) Repeated dose 28-day oral toxicity study in rodents	Exposure for 28 days At least 3 dose levels plus control At least 5 males and females per group Preferred rodent species: rat	Clinical observations Functional observations (4th exposure week – sensory reactivity to stimuli of different types, grip strength, motor activity) Body weight and food/water consumption Haematology (haematocrit, haemoglobin, erythrocyte count, reticulocytes, total and differential leucocyte count, platelet count, blood clotting time/potential). For suspected oxidisers add methaemoglobin concentration and Heinz bodies. Clinical biochemistry Urinalysis (optional) Oestrus cycle (optional) T3, T4, TSH (optional) Gross necropsy (full, detailed, all animals) Organ weights (all animals - liver, kidneys, adrenals, testes, epididymides, thymus, spleen, brain, heart, prostate, seminal vesicles with coagulating glands, uterus including cervix (optional), paired ovaries), thyroid Histopathology (full, at least control and high-dose groups - all gross lesions, brain, spinal cord, eye, stomach, small and large intestines, liver, kidneys, adrenals, spleen, heart, thymus, thyroid, trachea and lungs, gonads (testis and ovaries), accessory sex organs (uterus and cervix, epididymides, prostate + seminal vesicles with coagulating glands), vagina, urinary bladder, lymph nodes, peripheral nerve, skeletal muscle, bone marrow, optionally vaginal smears, male mammary glands, pituitary)
OECD TG 410 (1981) (EU B.9) Repeated dose dermal toxicity: 21/28-day study	Exposure for 21/28 days At least 3 dose levels plus control At least 5 males and females per group Rat, rabbit or guinea pig	Clinical observations Body weight and food/water consumption Haematology (haematocrit, haemoglobin, erythrocyte count, total and differential leucocyte count, clotting potential) Clinical biochemistry Urinalysis (optional) Gross necropsy (full, detailed, all animals) Organ weights (all animals - liver, kidneys, adrenals, testes) Histopathology (full, at least control and high-dose groups - all gross lesions, normal and treated skin, liver, kidney)

OECD TG 412 (EU B.8) Repeated dose inhalation toxicity: 28-day or 14-day study	Exposure for 28 or 14 days At least 3 concentrations plus control At least 5 males and females per group Rodents: preferred species rat	Clinical observations Body weight and food/water consumption Haematology (haematocrit, haemoglobin, erythrocyte count, total and differential leucocyte count, clotting potential) Clinical biochemistry Urinalysis (optional) Gross necropsy (full, detailed, all animals) Organ weights (all animals - liver, kidneys, adrenals, testes) Histopathology (full, at least control and high-dose groups - all gross lesions, lungs, liver, kidney, spleen, adrenals, heart)
OECD TG 408 (EU B.26) Repeated dose 90-day oral toxicity study in rodents	Exposure for 90 days At least 3 dose levels plus control At least 10 males and females per group Preferred rodent species: rat	Clinical observations Ophthalmological examination Functional observations (towards end of exposure period – sensory reactivity to stimuli of different types, grip strength, motor activity) Body weight and food/water consumption Haematology (haematocrit, haemoglobin, erythrocyte count, total and differential leucocyte count, platelet count, blood clotting time/potential) Clinical biochemistry Urinalysis Gross necropsy (full, detailed, all animals) Organ weights (all animals - liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain, heart) Histopathology (full, at least control and high-dose groups - all gross lesions, brain, spinal cord, pituitary, thyroid, parathyroid, thymus, oesophagus, salivary glands, stomach, small and large intestines, liver, pancreas, kidneys, adrenals, spleen, heart, trachea and lungs, aorta, gonads, uterus, accessory sex organs, female mammary gland, prostate, urinary bladder, gall bladder (mouse), lymph nodes, peripheral nerve, a section of bone marrow, and skin/eyes on indication)

OECD TG 409 (EU B.27) Repeated dose 90-day oral toxicity study in non-rodents	Exposure for 90 days At least 3 dose levels plus control At least 4 males and females per group Preferred species: dog	Clinical observations Ophthalmological examination Body weight and food/water consumption Haematology (as in TG 408) Clinical biochemistry Urinalysis Gross necropsy (full, detailed, all animals) Organ weights (as in TG 408 - additional: gall bladder, thyroid, parathyroid) Histopathology (as in TG 408 – additional: gall bladder, eyes)
OECD TG 411 (EU B.28) Subchronic dermal toxicity: 90-day study	Exposure for 90 days At least 3 dose levels plus control At least 10 males and females per group Rat, rabbit or guinea pig	Clinical observations Ophthalmological examination Body weight and food/water consumption Haematology (haematocrit, haemoglobin, erythrocyte count, total and differential leucocyte count, clotting potential) Clinical biochemistry Urinalysis Gross necropsy (full, detailed, all animals) Organ weights (all animals - liver, kidneys, adrenals, testes) Histopathology (full, at least control and high-dose groups - all gross lesions, normal and treated skin, and essentially the same organs and tissues as in TG 408)
OECD TG 413 (EU B.29) Subchronic inhalation toxicity: 90-day study	Exposure for 90 days At least 3 concentrations plus control At least 10 males and females per group Rodents: preferred species rat	Clinical observations Ophthalmological examination Body weight and food/water consumption Haematology (haematocrit, haemoglobin, erythrocyte count, total and differential leucocyte count, clotting potential) Clinical biochemistry Urinalysis Gross necropsy (full, detailed, all animals) Organ weights (all animals - liver, kidneys, adrenals, testes) Histopathology (full, at least control and high-dose groups - all gross lesions, respiratory tract, and essentially the same organs and tissues as in TG 408)

OECD TG 452 (EU B.30) Chronic toxicity studies	Exposure for at least 12 months At least 3 dose levels plus control Rodents : At least 20 males and females per group Non-rodents: At least 4 males and females per group Preferred rodent species: rat Preferred non-rodent species: dog	Clinical observations, including neurological changes Ophthalmological examination Body weight and food/water consumption Haematology (haematocrit, haemoglobin, erythrocyte count, total leucocyte count, platelet count, clotting potential) Clinical biochemistry Urinalysis Gross necropsy (full, detailed, all animals) Organ weights (all animals - brain, liver, kidneys, adrenals, gonads, thyroid/parathyroid (non-rodents only)) Histopathology (full, at least control and high-dose groups - all grossly visible tumours and other lesions, as well as essentially the same organs and tissues as in the 90-day studies (TG 408/409))
OECD TG 453 (EU B.33) Combined chronic toxicity / carcinogenicity studies	Exposure for at least 12 months (satellite groups) or majority of normal life span (carcinogenicity part) At least 3 dose levels plus control At least 50 males and females per group Satellite group: At least 20 males and females per group Preferred species: rat	Essentially as in TG 452

OECD TG 422[2] Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test	Exposure for a minimum of 4 weeks (males) or from 2 weeks prior to mating until at least post-natal day 13 (females – at least 9 weeks of exposure) At least 3 dose levels plus control At least 10 males and 12-13 females per group	Clinical observations as in TG 407 Functional observations as in TG 407 Body weight and food/water consumption Haematology as in TG 407 Hormonal measurements (thyroid hormone) Clinical biochemistry Urinalysis (optional) Gross necropsy (full, detailed, all adult animals) Organ weights (testes and epididymides - all males; liver, kidneys, adrenals, thymus, spleen, brain, heart, thyroid (optional) - in 5 animals of each sex per group, i.e. as in TG 407) Histopathology (ovaries, testes, epididymides, accessory sex organs, vagina, all gross lesions - all animals in at least control and high-dose groups; brain, spinal cord, eye, stomach, small and large intestines, liver, kidneys, adrenals, spleen, heart, thymus, thyroid (optional), trachea and lungs, urinary bladder, lymph nodes, peripheral nerve, a section of bone marrow - in 5 animals of each sex in at least control and high-dose groups, i.e. as in TG 407)
OECD TG 424 (EU B.43) Neurotoxicity study in rodents	Exposure for at least 28 days Dose levels: not specified At least 10 males and females per group Preferred rodent species: rat Generally oral route of administration	Detailed clinical observations Functional observations (sensory reactivity to stimuli of different types, grip strength, motor activity, more specialized tests on indication) Ophthalmological examination Body weight and food/water consumption Haematology (haematocrit, haemoglobin, erythrocyte count, total and differential leucocyte count, platelet count, blood clotting time/potential) Clinical biochemistry Histopathology: at least 5 animals/sex/group) for neuropathological examinations (brain, spinal cord, and peripheral nerves); remaining animals to be used either for specific neurobehavioural, neuropathological, neurochemical or electrophysiological procedures that may supplement the histopathology or alternatively, for routine pathological evaluations according to the guidelines for standard repeated dose toxicity studies

[2] To date there is no corresponding EU test method available.

OECD TG 419 (EU B.38) Delayed neurotoxicity of organophosphorus substances: 28-day repeated dose study	Exposure for 28 days At least 3 dose levels plus control At least 12 birds per group Species: domestic laying hen	Detailed clinical observations Body weight and food/water consumption Clinical biochemistry (NTE activity, acetylcholinesterase activity Gross necropsy (all animals) Histopathology (neural tissue)

- Other studies providing information on repeated dose toxicity:

Although not aiming at investigating repeated dose toxicity *per se*, other available OECD/EU test guideline studies involving repeated exposure of experimental animals may provide useful information on repeated dose toxicity. These studies are summarised in Table R.7.5–2.

It should be noted that the repeated dose toxicity studies, if carefully evaluated, may provide information on potential reproductive toxicity and on carcinogenicity (e.g. pre-neoplastic lesions).

The one- and two-generation studies (OECD TGs 415 and 416 / EU B.34 and B.35) and the extended one-generation reproductive toxicity study (OECD TG 443 / EU.B.56) may provide information on the general toxicological effects arising from repeated exposure over a prolonged period of time (about 90 days for parental animals) as clinical signs of toxicity, body weight, selected organ weights, and gross and microscopic changes of selected organs are recorded.

The prenatal developmental toxicity study (OECD TG 414 / EU B.31), the reproduction/developmental toxicity screening study (OECD TG 421[3]) and the developmental neurotoxicity study (draft OECD TG 426[3]) may give some indications of general toxicological effects arising from repeated exposure over a relatively limited period of the animals life span as clinical signs of toxicity and body weight are recorded.

The carcinogenicity study (OECD TG 451 / EU B.32) will, in addition to information on neoplastic lesions, also provide information on the general toxicological effects arising from repeated exposure over a major portion of the animal's life span as clinical signs of toxicity, body weight, and gross and microscopic changes of organs and tissues are recorded.

Table R.7.5–2 Overview of other *in vivo* test guideline studies giving information on repeated dose toxicity

Test	Design	Endpoints (general toxicity)
OECD TG 443 (EU B.56) Extended one-generation reproductive toxicity study	Exposure of 10 weeks (unless specific reasons to shorten) prior to mating (P) until post-natal day 90-120 (F1). If the extension of Cohort 1B is triggered, then until post-natal day 4 or 21 (F2). At least 3 dose levels plus control Sufficient mating pairs to	Clinical observations Body weight and food/water consumption Clinical chemistry Haematology, T4/TSH, urinalysis, splenic lymphocyte subpopulation analysis Gross necropsy (adults) Organ weights (reproductive organs, brain, liver, kidneys, heart, spleen, thymus, pituitary, thyroid, adrenal glands, lymph nodes proximal and distal to route of exposure and known target organs)

[3] To date there is no corresponding EU test method available.

	produce 20 animals per dose group (P generation), 20 mating pairs for extension of Cohort 1B, if triggered 10 males and females per dose group for Cohorts 2A, 2B, and/or 3, if triggered.	Histopathology (adults, full - at least for high-dose and control groups) Certain parameters for endocrine mode of action (Specific investigation on developmental neurotoxicity, in cases of a particular concern and/or immunotoxicity based on a particular concern)
OECD TG 416 (EU B.35) Two-generation reproduction toxicity study	Exposure before mating for at least one spermatogenic cycle until weaning of 2nd generation At least 3 dose levels plus control At least 20 parental males and females per group	Clinical observations Body weight and food/water consumption Gross necropsy (all parental animals) Organ weights (reproductive organs, brain, liver, kidneys, spleen, pituitary, thyroid, adrenal glands, and known target organs) Histopathology (reproductive organs, previously identified target organ(s) - at least control and high-dose groups
OECD TG 415 (EU B.34) One-generation reproduction toxicity Study	Exposure before mating for at least one spermatogenic cycle until weaning of 1st generation At least 3 dose levels plus control At least 20 parental males and females per group	As in TG 416
OECD TG 414 (EU B.31) Prenatal developmental toxicity study	Exposure at least from implantation to one or two days before expected birth At least 3 dose levels plus control At least 20 pregnant females per group	Clinical observations Body weight and food/water consumption Macroscopical examination all dams for any structural abnormalities or pathological changes, which may have influenced the pregnancy
OECD TG 421[4] Reproduction/ developmental toxicity screening test	Exposure of male animals for min. 4 weeks Exposure of dams from 2 weeks prior to mating until at least post-natal day 13[5] At least 3 dose levels plus control At least 8-10 parental males and females per group	Clinical observations Body weight and food/water consumption Gross necropsy (adult animals, special attention to reproductive organs) Hormonal measurements (thyroid hormone) Organ weights (all adult males: testes, epididymides, thyroid (optional)) Histopathology (reproductive organs and thyroid (optional) in at least control and high-dose groups)
OECD TG 426[4]	Exposure at least from	Clinical observations

[4] To date there is no corresponding EU test method available.

[5] OECD TG 421 was updated in 2015; according to the previous version of OECD TG 421, exposure was at least until post-natal day 4.

Developmental neurotoxicity study (draft)	implantation throughout lactation (PND 20) At least 3 dose levels plus control At least 20 pregnant females per group	Body weight and food/water consumption
OECD TG 451 (EU B.32) Carcinogenicity studies	Exposure for majority of normal life span At least 3 dose levels plus control At least 50 males and females per group	Clinical observations (special attention to tumour development) Body weight and food consumption Gross necropsy Histopathology (all groups - all grossly visible tumours or lesions suspected of being tumours; at least control and high-dose groups - brain, pituitary, thyroid, parathyroid, thymus, lungs, heart, salivary glands, liver, spleen, kidneys, adrenals, oesophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, uterus, urinary bladder, lymph nodes, pancreas, gonads, accessory sex organs, female mammary gland, skin, musculature, peripheral nerve, spinal cord, sternum with bone marrow and femur, eyes)

R.7.5.3.2 Human data on repeated dose toxicity

Human data adequate to serve as the sole basis for the hazard and dose-response assessment are rare. When available, reliable and relevant human data are preferable over animal data and can contribute to the overall *Weight of Evidence*. However, human volunteer studies are not recommended due to practical and ethical considerations involved in deliberate exposure of individuals to chemicals.

However the following types of human data may already be available:

- Analytical epidemiology studies on exposed populations. These data may be useful for identifying a relationship between human exposure and effects such as biological effect markers, early signs of chronic effects, disease occurrence, or long-term specific mortality risks. Study designs include case control studies, cohort studies and cross-sectional studies.

- Descriptive or correlation epidemiology studies. They examine differences in disease rates among human populations in relation to age, gender, race, and differences in temporal or environmental conditions. These studies may be useful for identifying priority areas for further research but not for dose-response information.

- Case reports describe a particular effect in an individual or a group of individuals exposed to a substance. Generally case reports are of limited value for hazard identification, especially if the exposure represents single exposures, abuse or misuse of certain substances.

- Controlled studies in human volunteers. These studies, including low exposure toxicokinetic studies, might also be of use in risk assessment.

- Meta-analysis. In this type of study data from multiple studies are combined and analysed in one overall assessment of the relative risk or dose-response curve.

R.7.5.3.3 Exposure considerations for repeated dose toxicity

Information on exposure, use and risk management measures should be collected in accordance with Article 10 and Annex VI (Section 3) of the REACH Regulation.

Such information may lead to an adaptation of the extent and nature of information needed on repeated dose toxicity under REACH; two types of *adaptations* are possible due to exposure considerations: exposure-based waiving of a study or exposure-based triggering of further studies.

More detailed guidance on exposure-based adaptations of the repeated dose toxicity information requirements is given in Sections 0 (Evaluation of available information) and R.7.5.6 (Integrated Testing Strategy).

Furthermore, the most appropriate route of administration needs to be considered taking into account human exposure.

R.7.5.4 Evaluation of available information on repeated dose toxicity

General guidance on how to evaluate the available information is given in Chapter R.4 of the *Guidance on IR&CSA*.

R.7.5.4.1 Non-human data on repeated dose toxicity

R.7.5.4.1.1 Non-testing data on repeated dose toxicity

Physico-chemical properties

The physico-chemical properties of a chemical substance under registration should always be considered before any new experimental *in vivo* repeated dose toxicity studies are undertaken.

The physico-chemical properties of a substance can indicate whether it is likely that the substance can be absorbed following exposure to a particular route and whether it (or an active metabolite) is likely to reach the target organ(s) and tissue(s). The physico-chemical properties are thus essential elements in deciding on the appropriate administration route to be applied in experimental *in vivo* repeated dose toxicity studies (see Section R.7.5.4.3).

The physico-chemical properties are also important in order to judge whether testing is technically possible. Testing for repeated dose toxicity may, as specified in Section 2 of Annex XI to the REACH Regulation, be omitted if it is technically not possible to conduct the study as a consequence of the properties of the substance (e.g. very volatile, highly reactive or unstable substances cannot be used, or mixing of the substance with water may cause danger of fire or explosion). Annex XI further emphasises that the guidance given in the test methods referred to in REACH Article 13(3), more specifically on the technical limitations of a specific method, must always be respected.

Additional generic guidance on the use of physico-chemical properties is provided for instance in Section R.7.12 on toxicokinetics, in Chapter R.7c of the *Guidance on IR&CSA*.

Read-across to structurally or mechanistically similar substances (SAR)

The grouping of substances and read-across offer a possibility for adaptation of the standard information requirements of the REACH Regulation. If the read-across approach is adequate, unnecessary testing can be avoided. A read-across approach can also support a conclusion for a REACH information requirement using a *Weight-of-Evidence* approach.

The application of the grouping concept means that REACH information requirements for physico-chemical properties, human health effects and/or environmental effects may be predicted from tests conducted on analogue substance(s) within the group, referred to as source substance(s), by interpolation (extrapolation is generally not recommended for predictions) to other substances in the group, referred to as target substance(s). This is called read-across.

The read-across approach has to be considered per information requirement due to the different complexities (e.g. key parameters, biological targets) of the studies needed to meet the information requirement. This means that read across (and the category approach) is specific for the property under consideration and therefore requires a specific read-across hypothesis and justification for predicting individual properties. In the case of repeated dose toxicity studies, the results obtained in repeated dose toxicity/reproductive toxicity screening studies, 28-day studies, 90-day studies or chronic studies may be predicted based on a read-across approach.

In the context of a grouping and read-across under REACH, adequate and reliable supporting evidence needs to be provided to substantiate scientific claims or hypotheses constituting the basis for predicting properties of a substance from data on another substance. Supporting evidence is not sufficient on its own to determine the property of the substance under consideration, but rather contributes to strengthening and justifying the read-across hypothesis.

There may be several lines of evidence used to justify read-across, with the aim of strengthening the case.

In principle, it is possible to predict the presence or absence of a property/effect by applying a read-across approach. For prediction of an absence of effect(s), typically no mechanistic insight is available to support such a claim. The absence of effect(s) may however be explained by other arguments, e.g. the absence of exposure of biological target(s) or a lack of biological interaction leading to an adverse outcome. These situations need to be addressed in the read-across hypothesis and read-across justification and should be supported by evidence.

The provisions of section 1.5 of Annex XI to the REACH Regulation require that the results of grouping and read-across approaches "should be adequate for the purpose of classification and labelling and/or risk assessment". Repeated-dose toxicity studies are typically used to derive C&L and DNELs on the basis of the strength of the observed effects (e.g. use the identified NOAEL as point of departure). For a prediction, this requires that the source study(ies) allow(s) for the identification of known value(s) of a property for one or more source substances which is then used to estimate the unknown value of the same property for the target substance. In this situation, it is essential to provide a robust scientific basis and quantitative supporting evidence (e.g. toxicokinetic information) to demonstrate that the type of effect and its strength observed in the source study can be used for C&L and/or risk assessment purposes for the target substance without under-estimating the property of the target substance under consideration.

Guidance on read-across is provided in Chapter R.6 "QSAR and grouping of chemicals" of the *Guidance on IR&CSA*. Further guidance can be found at: http://echa.europa.eu/support/grouping-of-substances-and-read-across.

ECHA has developed and published a Read-Across Assessment Framework (RAAF) to provide experts with a transparent and structured methodology to assess read-across approaches. The RAAF is available on ECHA's website at the above link.

Information on practical aspects of how to report read-across and/or category approaches in IUCLID is provided in the ECHA *Practical Guide 6* on "How to report read-across and categories".

(Q)SAR

A (Q)SAR analysis for a substance may give indications for a specific mechanism to occur and identify possible organ or systemic toxicity upon repeated exposure. The reliability, applicability and overall scope of (Q)SAR science to identify chemical hazard and assist in risk assessment have been evaluated by various groups and organisations. Guidance on this issue is presented in Section R.6.1 (in Chapter R.6 of the *Guidance on IR&CSA*) and in OECD Monograph No. 69 (OECD 2007b). Application of (Q)SARs should be documented according to the appropriate reporting formats: QSAR model reporting format (QMRF, see Section R.6.1.9) and QSAR prediction reporting format (QPRF, see Section R.6.1.10).

Overall, (Q)SAR approaches are currently not well validated for repeated dose toxicity and consequently no firm recommendations can be made concerning their routine use in a testing strategy in this area. There are a large number of potential targets/mechanisms associated with repeated dose toxicity that today cannot be adequately covered by a battery of (Q)SAR

models. Therefore, a negative result from current (Q)SAR models without other supporting evidence cannot be interpreted as demonstrating a lack of toxicological hazard or lack of a need for hazard classification. Another limitation of (Q)SAR modelling is that dose-response information, including the N(L)OAEL, is not provided. Similarly, a validated (Q)SAR model might identify a potential toxicological hazard, but because of limited confidence in this approach, such a result may not be adequate to support hazard classification.

In some cases, (Q)SAR results could be used as part of a *Weight-of-Evidence* approach, when considered alongside other data, provided the applicability domain is appropriate. Also, (Q)SAR data can be used as supporting evidence when assessing the toxicological properties by read-across within a substance grouping approach, providing the applicability domain is appropriate. Positive and negative (Q)SAR modelling results can be of value in a read-across assessment and for classification purposes.

R.7.5.4.1.2 Testing data on repeated dose toxicity

In vitro data

As mentioned earlier in Section R.7.5.3.1 available *in vitro* data are, at present, not useful on their own for regulatory decisions such as risk assessment and C&L. However, such data may be helpful in the assessment of repeated dose toxicity, for instance to detect local target organ effects and/or to clarify the mechanisms of action. Since, at present, there are no *in vitro* methods validated and accepted for regulatory purposes, the quality of each of these *in vitro* studies and the adequacy of the data provided should be carefully evaluated.

Generic guidance is given in Chapters R.4 and R.5 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, reproducibility of data and aspects such as vehicle, number of replicates, exposure/incubation time, GLP-compliance or comparable quality description.

Animal data

The basic concept of repeated dose toxicity studies to generate data on target organ toxicity following sub-acute to chronic exposure is to treat experimental animals for 4 weeks, 13 weeks or longer. These studies are mentioned in Section R.7.5.3.1 and summarised in Table R.7.5–1. In addition, other studies performed in experimental animals may provide useful information on repeated dose toxicity. While at present most alternative methods remain at the research and development stage and are not ready as surrogates for sub-chronic/chronic animal studies, there are opportunities to improve data collection for risk assessment providing greater efficiency and use of fewer animals and better use of resources. Although not required by REACH, other opportunities include early development of kinetic data, in conjunction with early repeated dose toxicity testing, thus ensuring that the maximum amount of information is drawn from the animal studies and for use in the risk assessment process.

The number of repeated dose toxicity studies available for a substance under registration is likely to be variable, ranging from none, a dose-range finding study, a 28-day repeated dose toxicity guideline study, to a series of guideline studies for some substances, including sub-chronic and/or chronic studies. There may also be studies employing different species and routes of exposure. In addition, special toxicity studies investigating further the nature, mechanism and/or dose-relationship of a critical effect in a target organ or tissue may also have been performed for some substances.

The following general guidance is provided for the evaluation of repeated dose toxicity data and the development of the *Weight of Evidence*:

- Studies on the most sensitive animal species should be selected as the significant ones, unless toxicokinetic and toxicodynamic data show that this species is less relevant for human risk assessment.

- Studies using an appropriate route, duration and frequency of exposure in relation to the expected route(s), frequency and duration of human exposure have greater weight.

- Studies enabling the identification of a NOAEL, and a robust hazard identification have a greater weight.

- Studies of a longer duration should be given greater weight than a repeated dose toxicity study of a shorter duration in the determination of the most relevant NOAEL.

- If sufficient evidence is available to identify the critical effect(s) (with regard to the dose-response relationship(s) and to the relevance for humans), and the target organ(s) and/or tissue(s), greater weight should be given to specific studies investigating this effect in the identification of a NOAEL. The critical effect can be a local as well as a systemic effect.

While data available from repeated dose toxicity studies not performed according to conventional guidelines and/or GLP may still provide information of relevance for risk assessment and C&L, such data require extra careful evaluation. Annex XI to the REACH Regulation specifically identifies circumstances where use of existing studies not carried out according to GLP or test methods referred to in Article 13(3) (guideline studies) can replace *in vivo* testing performed in accordance with REACH Article 13(3). Data from non-guideline studies must be considered to be equivalent to data generated by the corresponding test methods referred to in REACH Article 13(3) if the following conditions are met:

- adequacy for the purpose of C&L and/or risk assessment;

- adequate and reliable coverage of the key parameters foreseen to be investigated in the corresponding test methods referred to in REACH Article 13(3);

- exposure duration comparable to or longer than the corresponding test methods referred to in REACH Article 13(3) if exposure duration is a relevant parameter; and

- adequate and reliable documentation of the study is provided.

In all other situations, non-guideline studies may contribute to the overall weight of the evidence but they cannot stand alone for a hazard and risk assessment of a substance. Thus, such studies cannot serve as the sole basis for an assessment of repeated dose toxicity or for exempting from the standard information requirements for repeated dose toxicity at a given tonnage level, i.e. they cannot be used to identify a substance as being adequately controlled in relation to repeated dose toxicity.

If sufficient information from existing studies is available on the repeated dose toxicity potential of a substance in order to perform a risk assessment as well as to conclude on C&L under CLP for specific target organ toxicity arising from a repeated exposure (STOT-RE), no further *in vivo* testing is needed. The existing information is considered sufficient when, based on a *Weight-of-Evidence* analysis, the critical effect(s) and target organ(s) and tissue(s) can be identified, the dose-response relationship(s) and NOAEL(s) and/or LOAEL(s) for the critical effect(s) can be established, and the relevance for human beings can be assessed.

It should be noted that potential effects in certain target organs following repeated exposure may not be observed within the span of the 28-day study. Attention is also drawn to the fact that the protocols for the oral 28-day and 90-day studies include additional parameters compared to those for the 28-day and 90-day dermal and inhalation protocols.

Where it is considered that the existing data as a whole are inadequate for providing a clear assessment of this endpoint, the need for further testing should be considered in view of all available relevant information on the substance, including use pattern, the potential for human exposure, physico-chemical properties, and structural alerts. The testing strategy is presented in Section R.7.5.6.3.

Information from existing data on neurotoxicity or immunotoxicity or specific mode of action should be evaluated.

Regarding neurotoxicity and immunotoxicity, standard oral 28-day and 90-day toxicity studies include endpoints capable of detecting such effects. Indicators of neurotoxicity include clinical observations, a functional observational battery, motor activity assessment and histopathological examination of spinal cord and sciatic nerve. Indicators of immunotoxicity include changes in haematological parameters, serum globulin levels, alterations in immune system organ weights such as spleen and thymus, and histopathological changes in immune organs such as spleen, thymus, lymph nodes and bone marrow. Where data from standard oral 28-day and 90-day studies identify evidence of neurotoxicity or immunotoxicity, other studies may be necessary to further investigate the effects.

Additional guidance on immunotoxicity is available from the WHO/IPCS Guidance on Immunotoxicity for risk assessment (WHO, 2012).

More focus has also been put on endocrine disruptors. In relation to hazard and risk assessment, there are currently no test methods available that specifically detect all effects which have been linked to endocrine disruption mechanism. Guidance is available to facilitate the interpretation of hazard data derived from screens and tests in the OECD conceptual framework (see http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm#GD_Standardized_TG) has been pulished in 2012 (OECD, 2012).

Further Guidance on mode of action analysis is available from the WHO/IPCS framework on Mode of action and human relevance. The framework provides a structured and transparent approach to perform a *Weight-of-Evidence* analysis on mode of action (Meek *et al.*, 2014).

If data are not available from a standard oral 28-day repeated dose toxicity guideline study (OECD TG 407 / EU B.7), the minimum repeated dose toxicity data requirement (28-day study) at tonnage levels from 10 t/y may in certain circumstances be met by results obtained from *the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test* (OECD TG 422[6]). One advantage of this approach is to obtain information on repeated dose toxicity and reproductive toxicity in a single study, providing an overall saving in the number of animals used for testing. In addition, the number of animals is higher (10 per sex compared to 5 per sex in the standard oral 28-day study)[7] and the dosing period is longer in the combined study than in the standard oral 28-day study. Therefore, more information on repeated dose toxicity could be expected from the combined study. Potential complications in using the combined study include the selection of adequate dose levels to examine adequately both repeated dose toxicity and reproductive toxicity. In addition, interpretation of the results may be complicated due to differences in sensitivity between pregnant and non-pregnant animals, and an assessment of the general toxicity may be more difficult especially when serum and histopathological parameters are not evaluated at the same time in the study. Consequently, where the combined study is used for the assessment of repeated dose toxicity, the use of data obtained from such a study should be clearly indicated. Despite such

[6] To date there is no corresponding EU test method available.

[7] Histopathological examination of reproductive organs and of all organs showing macroscopic lesions is required for all adult animals. All other organs are investigated in 5 animals per sex and dose.

complications, the use of the combined study is recommended for the initial hazard assessment of the repeated dose toxicity potential of a substance when this study is also relevant for reproductive toxicity assessment.

In general, results from toxicological studies requiring repeated administration of a test substance (see also Section R.7.5.3.1) such as *reproduction and developmental toxicity studies* can contribute to the assessment of repeated dose toxicity. However, such toxicological studies rarely provide the information obtained from a standard repeated dose toxicity study and, therefore, cannot stand alone as the sole basis for the assessment of repeated dose toxicity or for exempting from the standard information requirements for repeated dose toxicity at a given tonnage level.

Studies such as *acute toxicity, in vivo irritation* as well as *in vivo genotoxicity studies* contribute limited information to the overall assessment of the repeated dose toxicity. However, such studies may be useful in deciding on the dose levels for use in repeated dose toxicity.

Guidance on the dose selection for repeated dose toxicity testing (see also Table R.7.5–1) is provided in detail in the EU and OECD test guidelines. Unless limited by the physico-chemical properties or biological effects of the test substance, the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering.

Although not required by REACH, toxicokinetic studies may be helpful in the evaluation and interpretation of repeated dose toxicity data, for example in relation to accumulation of a substance or its metabolites in certain tissues or organs as well as in relation to mechanistic aspects of repeated dose toxicity and species differences. Toxicokinetic information can also be used in the selection of the dose levels. When conducting repeated dose toxicity studies it is necessary to ensure that the observed treatment-related toxicity is not associated with the administration of excessive high doses causing saturation of absorption and detoxification mechanisms. The results obtained from studies using excessive doses causing saturation of metabolism are often of limited value in defining the risk posed at more relevant and realistic exposure levels where a substance can be readily metabolised and cleared from the body. It is suggested that a key element in designing better repeated dose toxicity studies is to select appropriate dose levels based on results from useful metabolic and toxicokinetic investigations. Further details on the application of toxicokinetic information in the design and evaluation of repeated dose toxicity studies is available in Section R.7.12 on toxicokinetics, in Chapter R.7c of the *Guidance on IR&CSA*.

R.7.5.4.2 Human data on repeated dose toxicity

Human data in the form of epidemiological studies or case reports can contribute to the hazard identification process as well as to the risk assessment process itself. Criteria for assessing the adequacy of epidemiological studies include an adequate research design, proper selection and characterisation of the exposed and control groups, adequate characterisation of exposure, sufficient duration of follow-up for the disease to develop as an effect of the exposure, valid ascertainment of effect, proper consideration of bias and confounding factors, proper statistical analysis and reasonable statistical power to detect an effect. These types of criteria have been described in more detail by Swaen (2006) and can be derived from Epidemiology Textbooks (Checkoway *et al.*, 1989; Hernberg, 1991; Rothman, 1998).

The results from human experimental studies are often limited by a number of factors, such as a relatively small number of subjects, short duration of exposure, and low dose levels resulting in poor sensitivity in detecting effects.

In relation to hazard identification, the relative lack of sensitivity of human data may cause particular difficulty. Therefore, negative human data cannot be used to override the positive findings in animals, unless it has been demonstrated that the mode of action of a certain toxic

response observed in animals is not relevant for humans. In such a case a full justification is required. It is emphasised that testing with human volunteers is strongly discouraged, but when there are good quality data already available they can be used in the overall *Weight of Evidence*.

R.7.5.4.3 Exposure considerations for repeated dose toxicity

R.7.5.4.3.1 Adaptations

Two types of *adaptations* from testing are possible due to exposure considerations: exposure-based waiving of a study and exposure-based triggering of further studies. More information on exposure-based waiving is available in Section R.5.1 in Chapter R.5 of the *Guidance on IR&CSA*. More detailed guidance on exposure-based adaptations of the testing requirements for repeated dose toxicity is given below and in Section R.7.5.6 (Integrated Testing Strategy).

R.7.5.4.3.2 Most appropriate route

Concerning repeated dose toxicity testing the oral route is the preferred one. However, dependent on the physico-chemical properties of a substance as well as on the most relevant route of human exposure, the dermal or the inhalation route could also be appropriate as specified in Annexes VIII and IX to the REACH Regulation.

The dermal route is appropriate if the physico-chemical properties suggest a potential for a significant rate of absorption through the skin and the criteria provided in Section 8.6.1 of Annex VIII and/or column 2 of Section 8.6.2 in Annex IX to the REACH Regulation for the appropriateness of testing by the dermal route are fulfilled.

The inhalation route is appropriate if exposure of humans *via* inhalation is the most relevant route of human exposure taking into account the vapour pressure of the substance and/or the possibility of exposure to aerosols, particles or droplets of an inhalable size.

If more than one route is appropriate, a decision on the most appropriate route of administration is required (see also Section R.7.5.6.3.4, under "Selection of the most appropriate route of administration").

R.7.5.4.3.3 Requirement for further studies

According to Annexes VIII-X to the REACH Regulation further studies must be proposed by the registrant or may be required by the Agency for example if there is particular concern regarding exposure, e.g. use in consumer products leading to exposure levels which are:

- close to the dose levels at which toxicity to humans may be expected (Annex VIII) ,i.e. a dose lower than, but in the vicinity of, the dose levels at which toxicity to humans may be expected;
- high relative to the dose levels at which toxicity to humans may be expected (Annex IX), i.e. exposure levels higher than the dose levels at which toxicity to humans may be expected;
- close to the dose levels at which toxicity is observed (Annex X), i.e. a dose lower than, but in the vicinity of, the dose levels at which toxicity is observed from animal studies.

Any of the exposure-triggered studies proposed by the registrant or required by the Agency should be considered on a case-by-case basis.

R.7.5.4.3.4 Waiving of repeated dose toxicity studies

Various types of exposure considerations are a possible basis for the *waiving* of repeated dose toxicity studies. For instance, it is stated in REACH Article 13 and Section 3 of Annex XI that testing in accordance with Sections 8.6 and 8.7 (i.e. repeated dose toxicity and reproductive toxicity) of Annex VIII and with Annexes IX and X may be omitted based on the exposure scenario(s) developed in the Chemical Safety Report. Adequate justification and documentation must in all cases be provided (see Section R.5.1 in Chapter R.5 of the *Guidance on IR&CSA*).

Further, the sub-chronic toxicity study (90-day study) does not need to be conducted according to Annex IX to the REACH Regulation 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*". In order to omit the study the prerequisites interpreted above have to be considered jointly since the word "*and*" is used in between them. In addition, limited human exposure would strengthen the possibility for waiving.

The term "*unreactive*" in the above quotation from the legal text can relate to the inherent chemical reactivity and, as such, can be interpreted as an indicator of the lack of local effects and mutagenicity. The terms "*insoluble and not inhalable*" can be interpreted as indicators of low exposure potential and should be further defined. The terms "*no evidence of absorption*" imply that there has to be some evidence of the lack of absorption in order to omit the study. Further, "*no evidence of toxicity in a 28-day limit test*" can be interpreted as meaning that there has to be at least a 28-day limit test available in order to waive the 90-day study, and this 28-day study should not show any sign of toxicity at a dose of 1000 mg/kg bw.

Interpretation of "limited exposure" should encompass the level of exposure, the frequency and/or the duration of exposure. Therefore, "limited exposure" must be considered on a case-by-case basis.

Finally, according to Annex VIII to the REACH Regulation, testing for repeated dose toxicity (28-day study) does not need to be conducted if "*relevant human exposure can be excluded*".

Relevant human exposure depends on the inherent properties of the substance, if the population comes into contact with the substance or not, and how the substance is used. Thus, waiving might be considered on a case-by-case basis.

The concept of Threshold of Toxicological Concern (TTC) might be applied to reduce the use of animals and other evaluation resources (Kroes *et al*., 2004). Use of the TTC concept may also be seen as a driving force for deriving exposure information of adequate quality. However, there are a number of limitations or drawbacks that should be taken into consideration in deciding if the concept is to be applied for industrial chemicals and further discussions on the cut-off values are needed before integration into into the guidance (see Appendix R.7-1 to Chapter R.7, in Chapter R.7c of the *Guidance on IR&CSA*; TemaNord, 2005).

R.7.5.4.4 Remaining uncertainty on repeated dose toxicity

The key requirement for a CSA is the DNELs per exposure scenario (box 5 of Figure R.7.5–1). The DNEL for repeated dose toxicity is the threshold of the critical effect derived in a *Weight-of-Evidence* assessment of the available repeated dose toxicity data, to which is associated an overall assessment factor (AF) that takes into account any uncertainty. The following elements contribute to the uncertainty in determination of a threshold for the critical effects and the selection of the AF (further guidance on deriving a DNEL and application of AFs is provided in Chapter R.8 of the *Guidance on IR&CSA*).

R.7.5.4.4.1 Threshold of the critical effect

In the determination of the overall threshold for repeated dose toxicity all relevant information is evaluated to determine the lowest dose that induces an adverse effect (i.e. LOAEL or LOAEC) and the highest level with no biologically or statically significant adverse effects (i.e. NOAEL or NOAEC). In this assessment all toxicological responses are taken into account and the critical effect is identified. The uncertainty in the threshold depends on the strength of the data and is largely determined by the design of the underlying experimental data. Parameters such as group size, study type/duration or the methodology need to be taken into account in the assessment of the uncertainty in the threshold of the critical effect(s).

The NOAEL is typically used as the starting point for the derivation of the DNEL. In case a NOAEL has not been achieved, a LOAEL may be used, provided the available information is sufficient for a robust hazard assessment and for C&L. The Benchmark Dose (BMD) may also be used as the starting point for the derivation of the DNEL (see Chapter R.8 of the *Guidance on IR&CSA*).

The selection of NOAEL or LOAEL is usually based on the dose levels used in the most relevant toxicity study, without considering the shape of the dose-response curve. Therefore, the NOAEL/LOAEL may not reflect the true threshold for the adverse effect. On the other hand, the BMD is a statistical approach for the determination of the threshold and relies on the dose-response curve. Alternatively, mathematical curve fitting techniques or statistical approaches exist to determine the threshold for an adverse effect. The use of such approaches (e.g. Benchmark Dose) to estimate the threshold should be considered on a case-by-case basis. For further guidance see Chapter R.8 of the *Guidance on IR&CSA*.

R.7.5.4.4.2 Overall AF

Variability in sensitivity across and within species is another source of uncertainty for repeated dose toxicity. These inter- and intraspecies differences, respectively, are linked with variations in the toxicokinetics and dynamics of a substance. Information derived from non-testing, *in vitro* or *in vivo* methods may lead to an improvement of the understanding of the relevance of animal data for human risk assessment and may lead to a replacement of adopted standard default AFs for these differences.

The quality of the whole database should be assessed for reliability and consistency across different studies and endpoints and take into account the quality of the testing method, size and power of the study design, biological plausibility, dose-response relationships and statistical association. Missing test data might be substituted by non-testing data obtained from physico-chemical properties, read-across to structurally or mechanistically related substances (SAR/chemical category). (Q)SAR predictions could also provide information to be used as part of a *Weight-of-Evidence* approach (for more details on (Q)SAR models for Repeated Dose Toxicity see Appendix R.7.5-2). *In vitro* data as well as non-standard *in vivo* tests might be used to fill in data gaps. Such data in combination with toxicity tests according to standard OECD/EU guidelines may in some cases lead to an improved understanding of the toxicological effect resulting in a reduction in the overall uncertainty. On the other hand information solely based on *in vitro* and non-testing data is at present insufficient to be used as a surrogate for repeated dose toxicity data and the uncertainty is sufficiently high that such information is unsuitable for use in a CSA and for C&L. In the case of chemical categories, information from non-testing methods or *in vitro* data may be used to fulfil the data requirements for repeated dose toxicity and lead to improvement in the overall reliability and consistency for the read-across within a category of substances.

Since the adequacy and/or completeness of different data may vary, lack of quality and completeness of the overall database should be compensated for by an assessment factor to cover for the remaining uncertainty.

Besides AFs addressing these differences (inter- and intraspecies, quality of the whole database), other uncertainties relating to differences between human and animal exposure conditions (e.g. route and duration), and dose-response characteristics are described in the more extensive guidance on deriving a DNEL (see Section R.8.4.3 in Chapter R.8 of the *Guidance on IR&CSA*).

R.7.5.4.4.3 Other considerations

Another situation may arise when testing is not technically possible, a waiving option indicated in Section 2 of Annex XI to the REACH Regulation (see also Chapter R.5 of the *Guidance on IR&CSA*). In such cases, approaches such as QSAR, category formation and read-across may be helpful in the hazard characterisation. These approaches should also be considered for generating information that might be suitable as a surrogate for a dose descriptor. Alternatively, generic threshold approaches, e.g. TTC, might be considered for defining the starting point of a risk characterisation (see Appendix R.7-1 to Chapter R.7, in Chapter R.7c of the *Guidance on IR&CSA*).

R.7.5.5 Conclusions on repeated dose toxicity

The evaluation of all available toxicological information for repeated dose toxicity (step 3 in Figure R.7.5–1) should include an assessment whether the available information as a whole (i.e. testing and non-testing, and relevant information from studies addressing other endpoints) meets the tonnage-driven data requirements necessary to fulfil the REACH requirements. A *Weight-of-Evidence* approach should be used in assessing the database for a substance. This approach requires a critical evaluation of the entire body of available data for consistency and biological plausibility. Potentially relevant studies should be judged for quality and studies of high quality given more weight than those of lower quality. When both epidemiological and experimental data are available, similarity of effects between humans and animals is given more weight. If the mechanism or mode of action is well characterised, this information is used in the interpretation of observed effects in either human or animal studies. *Weight of Evidence* is not to be interpreted as simply tallying the number of positive and negative studies, nor does it imply an averaging of the doses or exposures identified in individual studies that may be suitable as starting points for risk assessment. The study or studies used for the starting point are identified by an informed and expert evaluation of all the available evidence.

The available repeated dose toxicity data should be evaluated in detail for a characterisation of the health hazards upon repeated exposure. In this process an assessment of all toxicological effects, their dose-response relationships and possible thresholds are taken into account. The evaluation should include an assessment of the severity of the effect, whether the observed effect(s) is (are) adverse or adaptive, reversible or irreversible, or precursor to a more significant effect or secondary to general toxicity. Correlations between changes in several parameters, e.g. between clinical or biochemical measurements, organ weights and (histo)pathological effects, will be helpful in the evaluation of the nature of effects. Further guidance to this issue can be found in publications of the International Programme on Chemical Safety (IPCS, 1994; 1999) and ECETOC (2002).

The effect data are also analysed for indications of potential serious toxicity of target organs or specific organ systems (e.g. neurotoxicity or immunotoxicity), delayed effects or cumulative toxicity. Furthermore, the evaluation should take into account the study details and determine if the exposure conditions and duration and the parameters studied are appropriate for an adequate characterisation of the toxicological effect(s).

If an evaluation allows the conclusion that the information of the repeated dose toxicity is adequate for a robust characterisation of the toxicological hazards, including an estimate of a dose descriptor (NOAEL/LOAEL/BMD), and the data are adequate for risk assessment and C&L, no further testing is necessary unless there are indications for further risk, according to column 2 of Annexes VIII-X to the REACH Regulation.

Another consideration to be taken into account is whether the study duration has been appropriate for an adequate expression of the toxicological effects. If the critical effect involves serious specific system or target organ toxicity (e.g. haemolytic anaemia, neurotoxicity or immunotoxicity), delayed effects or cumulative toxicity and a threshold has **not** been established, then dose extrapolation may not be appropriate and further studies are required. In this case a specialised study is likely to be more appropriate for an improved hazard characterisation and should be considered instead of a standard short-term rodent or sub-chronic toxicity test at this stage.

In the identification of a NOAEL, other factors need to be considered such as the severity of the effect, presence or absence of a dose- and time-effect relationship and/or a dose- and time-response relationship, biological relevance, reversibility, and normal biological variation of an effect that may be shown by representative historical control values (IPCS, 1990).

R.7.5.5.1 Concluding on suitability for Classification and Labelling

According to REACH, the data used (existing or generated) should be adequate for the purposes of C&L and risk assessment. Therefore, the data should allow a comparison with the criteria for STOT-RE classification (box 4 in Figure R.7.5–1). These criteria focus on the strength and severity of the effects and the dose levels at which they occur related to the classification categories.

Basically the following conclusions can be obtained from the assessment of adequacy for C&L for repeated dose toxicity:

- Data are considered adequate for the purpose of C&L if they allow a comparison against the criteria for STOT-RE classification under CLP (boxes 6 and 11 in Figure R.7.5–1)[8].

- Data are considered as inadequate for the purpose of C&L and cannot be checked against the CLP criteria (inconclusive or lacking data). In this case testing should be considered.

For further details, see Section 3.9 of the *Guidance on the Application of the CLP criteria*.

R.7.5.5.2 Concluding on suitability for Chemical Safety Assessment

In order to be suitable for CSA (box 5 of Figure R.7.5–1) appropriate DNELs have to be established for each exposure scenario. Typically, the derivation of the DNEL takes into account a dose descriptor, modification of the starting point and application of assessment factors (see Chapter R.8 of the *Guidance on IR&CSA*).

[8] It should be noted that although the exposure assessment and risk characterisation do not need to be performed when a substance is not classified (see Part A, section A.1.2 of the *Guidance on IR&CSA*), for potency-based endpoints like repeated dose toxicity there could still potentially be a risk. Therefore one might consider performing an exposure assessment and risk characterisation on a voluntary basis, to ensure safe handling and use.

For the identification of the so-called dose descriptor an appropriate threshold dose for the critical effect should be established as the starting point for DNEL derivation, i.e. a NOAEL or BMD. If a NOAEL can not be identified, the LOAEL may be used instead provided the data are adequate for a robust hazard assessment.

It is to be noted that the dose descriptor should be route-specific. Thus, in case only animal data with oral exposure are available and humans are exposed mainly *via* skin and/or inhalation, a DNEL for dermal route and/or DNEL for inhalation route are needed: i.e. route-to-route extrapolation is needed, if allowed. Guidance for this route-to-route extrapolation is provided in Section R.8.4.2 in Chapter R.8 of the *Guidance on IR&CSA*.

If this route-to-route extrapolation is not allowed, route-specific information is needed, possibly including testing, as a last resort (see Section R.7.5.6.3).

Derivation of a DNEL from this dose descriptor by applying AFs (to address uncertainty in the available data) is described elsewhere (see Section R.8.4.3 in Chapter R.8 of the *Guidance on IR&CSA*; see also Section R.7.5.4.4).

R.7.5.5.3 Information not adequate

A *Weight of Evidence* 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 order to proceed in further information gathering the testing strategy described in Section R.7.5.6.3 can be adopted.

R.7.5.6 Integrated Testing Strategy (ITS) for repeated dose toxicity

R.7.5.6.1 Objective / General principles

The objective in this testing strategy is to give guidance on a stepwise approach to hazard identification with regard to repeated dose toxicity (Figure R.7.5–1).

A principle of the strategy is that the results of one study are evaluated before another study is initiated. The strategy seeks to ensure that the data requirements are met in the most efficient and humane manner so that animal usage and costs are minimised.

The core objectives of the Integrated Testing Strategy (ITS) for repeated dose toxicity are to generate sufficient information to allow:

- Characterisation of the hazard profile and the dose-response of a substance upon repeated exposure;
- Performance of a chemical safety assessment for repeated dose toxicity.

Information generated in this strategy should be suitable for C&L according to the criteria given in Annex I to the CLP Regulation.

In addition, information from repeated dose toxicity studies can give valuable information for other endpoints based on repeated exposure (e.g. reproductive and developmental toxicity), and are valuable for other *in vivo* studies.

R.7.5.6.2 Preliminary considerations

On the basis of the objectives outlined above, a framework has been developed so that informed decisions can be made on the need for further testing. If generation of further data is

deemed necessary, the information needs should be met efficiently in terms of resources and animal use. This means using the most appropriate study type in accordance with the tonnage-driven requirements stipulated by the REACH information requirements and taking into account modifications due to considerations of exposure, grouping and category formation. The data requirements may be increased or decreased taking into account exposure considerations or the level of concern noted during any of the stages in the testing strategy.

Testing for repeated dose toxicity is not required for chemicals produced at tonnage levels less than 10 tonnes per annum (t/y). At higher production volumes, standard data requirements are, in general, increased with each tonnage band (see Section R.7.5.2). Maintaining flexibility to adopt the most appropriate testing regime for any single chemical is a key component of the ITS. However, regardless of whether testing for repeated dose toxicity is required or not at a specific tonnage level, all existing test data and all other available and relevant information on the substance should be collected.

In the previous Section R.7.4, the possibility to use a sub-acute oral toxicity study to adapt the information requirement for the acute oral toxicity has been addressed. This adaptation may be proposed when the NOAEL from the sub-acute study is above 1000 mg/kg and when low acute toxicity can be supported by some additional information, which should then be used in a *Weight-of-Evidence* approach. In case a registrant has some indications that a substance is of low toxicity and he intends to "waive" the acute oral toxicity study, he should perform the sub-acute oral toxicity study first, i.e. before the acute oral study. Detailed guidance on this *Weight-of-Evidence* based adaptation of the acute oral toxicity study is given in Appendix R.7.4-1 to Section R.7.4.

R.7.5.6.3 Testing strategy for repeated dose toxicity

In order to proceed in further information gathering the following testing strategy is outlined (step 4 in Figure R.7.5–1).

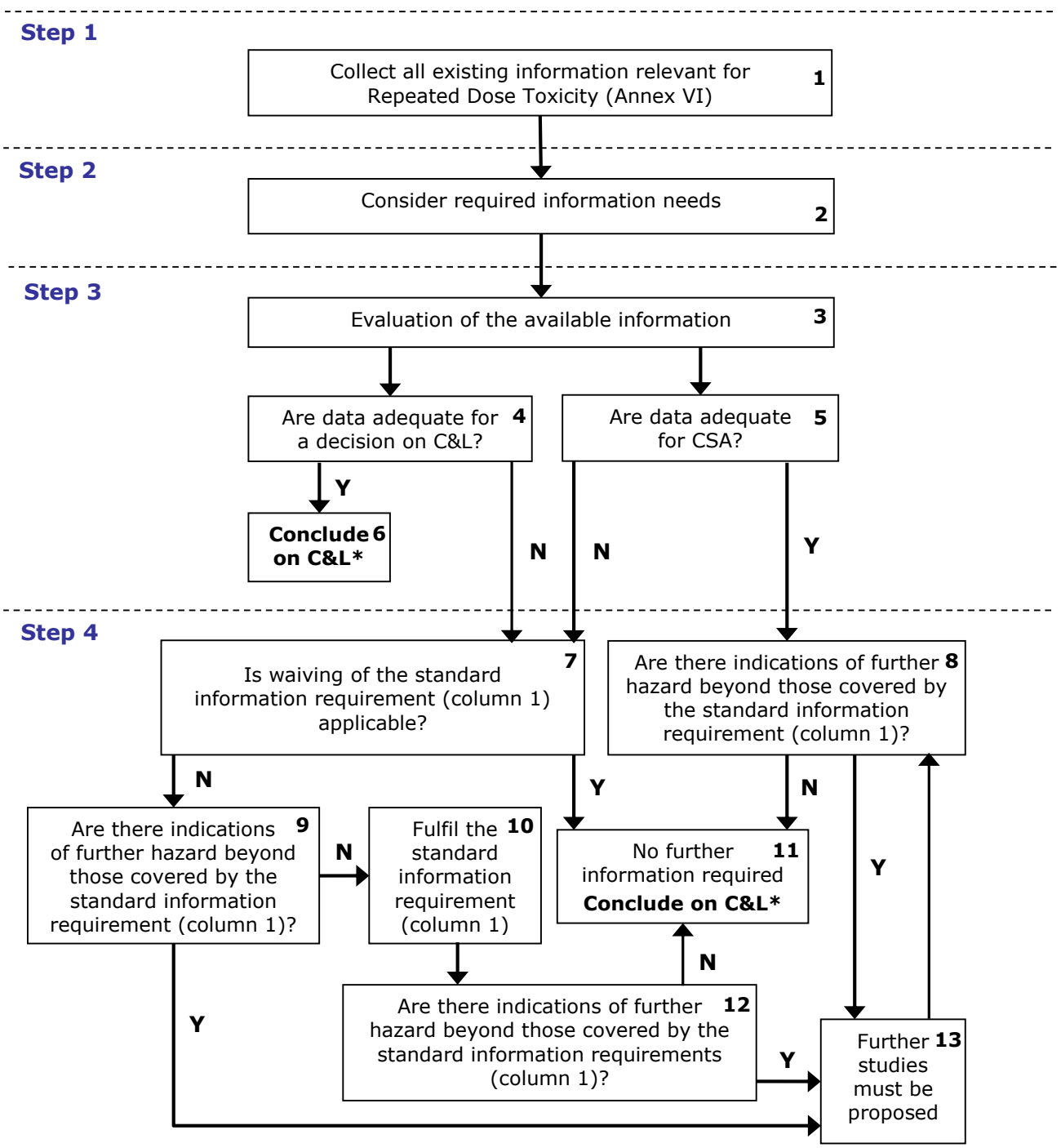
Before testing is initiated the available information should be scrutinised for evidence that may indicate severe effects, serious specific system or target organ toxicity (e.g. neurotoxicity or immunotoxicity), delayed effects or cumulative toxicity (boxes 8, 9 and 12 in Figure R.7.5–1). These indications may provide a trigger for specialised study protocols instead of the standard protocols for the short-term and/or (sub)chronic toxicity (box 13 in Figure R.7.5–1). These specific protocols should be designed on a case-by-case basis, such that they enable an adequate characterisation of these hazards, including the dose-response, threshold for the toxic effect and an understanding of the nature of the toxic effects. An example of such an approach is given in Appendix R.7.5-1.

Annexes VII-X to the REACH Regulation provide the standard information requirements in Column 1 (box 10 of Figure R.7.5–1) and specify triggering and waiving possibilities for the specific endpoints in Column 2. Different descriptors used for repeated dose toxicity in these annexes varying from *limited* (Annex IX) to *no relevant exposure* (Annex VIII). In addition, Annex XI to the REACH Regulation contains basic approaches, or rules for adaptation of the standard testing regime, set out in Annexes VII-IX (see Chapter R.5 of the *Guidance on IR&CSA*; for waiving see box 7 in Figure R.7.5–1).

Exposure considerations at this stage may trigger a need for additional data if the applications include wide dispersive uses to a large population (e.g. consumer products) and if a particular concern exists for a low margin of exposure (box 13 in Figure R.7.5–1). The data to be generated at this stage should aim at improving the risk quotient and could therefore be a trigger for an improved exposure characterisation or an improved hazard characterisation. In the latter case the required information might include a special study leading to an improved characterisation of the critical toxic endpoint thereby decreasing the uncertainty in the NOAEL

for repeated dose toxicity. An example of such a testing approach applied to neurotoxicity is given in Appendix R.7.5-1.

Figure R.7.5–1 Integrated Testing Strategy for repeated dose toxicity



Utilisation of the different tests at each of the different tonnage levels is summarised below. It should be noted that the latest update of a test guideline (OECD TG and/or EU method) should be used for conducting new tests. In addition Section R.7.5.6.3.4 should be considered before deciding on the test design for repeated dose toxicity assessment.

R.7.5.6.3.1 10 t/y or more (Annex VIII to the REACH Regulation)

At this tonnage level a short-term (28-day) toxicity test (OECD TG 407 / EU B.7) is usually required. The use of a combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD TG 422[9]) is recommended if an initial assessment of repeated dose toxicity and reproductive toxicity is required. The route of exposure in these tests is oral unless the predominant route of human exposure or the physico-chemical properties indicate that the dermal or inhalational route may be a more appropriate route of exposure to assess the repeated dose toxicity test (requiring OECD TG 410 or 412 / EU B.9 or B.8).

If the results of a short-term rodent toxicity study (OECD TGs 407, 410, 412, 422) are adequate for dose-response characterisation, C&L and risk assessment, and if there are no indications for further risks, no further testing is required (see Section R.7.5.5.2 for a detailed discussion of the criteria for a robust hazard characterisation).

At this tonnage level the short-term toxicity study (28 days) does not need to be conducted if:

- a reliable sub-chronic (90 days) or chronic toxicity study is available, provided that an appropriate species, dosage, and route of administration were used; or
- where a substance undergoes immediate disintegration and there are sufficient data on the cleavage products; or
- relevant human exposure can be excluded in accordance with Annex XI Section 3.

It should be noted that any of the rules for adaptation according to Annex XI also applies (see Chapter R.5 of the *Guidance on IR&CSA*). For further details see Section R.7.5.6.3.5 on Annex XI below.

According to REACH (Annex IX, 8.6.2), the sub-chronic toxicity study (90 days) must be proposed by the registrant if:

- the frequency and duration of human exposure indicates that a longer term study is appropriate;

and one of the following conditions is met:

- other available data indicate that the substance may have a dangerous property that cannot be detected in a short-term toxicity study; or
- appropriately designed toxicokinetic studies reveal accumulation of the substance or its metabolites in certain tissues or organs which would possibly remain undetected in a short-term toxicity study but which are liable to result in adverse effects after prolonged exposure.

REACH also specifies that further studies must be proposed by the registrant or may be required by the Agency in accordance with Article 40 or 41 in case of:

- failure to identify a NOAEL in the 28 or the 90 days study, unless the reason for the failure to identify a NOAEL is absence of adverse toxic effects; or
- toxicity of particular concern (e.g. serious/severe effects); or

[9] To date there is no corresponding EU test method available.

- indications of an effect for which the available evidence is inadequate for toxicological and/or risk characterisation. In such cases it may also be more appropriate to perform specific toxicological studies that are designed to investigate these effects (e.g. immunotoxicity, neurotoxicity); or

- the route of exposure used in the initial repeated dose study was inappropriate in relation to the expected route of human exposure and route-to-route extrapolation cannot be made; or

- particular concern regarding exposure (e.g. use in consumer products leading to exposure levels which are close to the dose levels at which toxicity to humans may be expected); or

- effects shown in substances with a clear relationship in molecular structure with the substance being studied, were not detected in the 28 or the 90 days study.

It should be pointed out that a failure to identify a NOAEL does not lead to a data gap in every case and should not trigger additional studies by default. If the data are sufficient for a robust hazard assessment and for C&L, the LOAEL may be used as the starting point for the CSA (see also Sections R.7.5.4.4 and R.7.5.5 and Chapter R.8 of the *Guidance on IR&CSA*).

A specialised study is likely to be more appropriate for an improved hazard characterisation and should be considered instead of a standard short-term rodent or sub-chronic toxicity test at this stage.

R.7.5.6.3.2 100 t/y or more (Annex IX to the REACH Regulation)

At this tonnage level, the following information is required (REACH Annex IX, Sections 8.6.1 and 8.6.2):

- a short-term study (28 days) in a single rodent species is the minimum requirement. The preferred route of exposure in these tests is oral (OECD TG 407 / EU B.7; TG 422[10]) unless the predominant route of human exposure or the physico-chemical properties indicate(s) that the dermal or inhalation route (OECD TGs 410, 412 / EU B.9, B.8) is a more appropriate route of exposure in the repeated dose toxicity tests.

- a sub-chronic toxicity study (90 days) in a single rodent species is usually required. The preferred route of exposure in these tests is oral (OECD TG 408 / EU B.26) unless the predominant route of human exposure or the physico-chemical properties indicate(s) that the dermal or inhalation route (OECD TGs 411, 413 / EU B.28, B.29) is a more appropriate route of exposure in the repeated dose toxicity tests.

According to REACH, at this tonnage level the sub-chronic toxicity study (90 days) does not need to be conducted if:

- a reliable short-term toxicity study (28 days) is available showing severe toxicity effects according to the criteria for classifying the substance as STOT-RE, for which the observed NOAEL-28 days, with the application of an appropriate assessment factor, allows the extrapolation towards the NOAEL-90 days for the same route of exposure; or

- a reliable chronic toxicity study is available, provided that an appropriate species and route of administration were used; or

[10] To date there is no corresponding EU test method available.

- a substance undergoes immediate disintegration and there are sufficient data on the cleavage products (both for systemic effects and effects at the site of uptake); or

- 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.

It should be noted that any of the rules for adaptation according to Annex XI also applies. For further details see Section R.7.5.6.3.5 on Annex XI below.

In case human exposure is limited or different in frequency and duration from that used in the test protocol for repeated dose toxicity, the sub-chronic toxicity study may not be necessary if the data for the short-term toxicity study are adequate for a robust hazard characterisation, a risk assessment and classification and labelling (C&L). This adaptation requires full justification by the registrant.

In case the *Weight of Evidence* indicates that the available information is adequate to characterise the short-term toxicity and sufficiently robust for proper dose-selection of the 90-day study, a dedicated 28-day study is not necessary at this stage.

No further testing is required if the available data, which may include a sub-chronic rodent toxicity study (OECD TGs 408, 411, 413 / EU B.26, B.28, B.29) are adequate for a dose response characterisation and C&L and risk assessment.

In case data are inadequate for hazard characterisation and risk assessment further studies must be proposed by the registrant or may be required by the Agency in accordance with REACH Articles 40 or 41: according to REACH Annex IX Section 6.6.2 such a situation may arise if there is:

- failure to identify a NOAEL in the 90 days study unless the reason for the failure to identify a NOAEL is absence of adverse toxic effects; or

- toxicity of particular concern (e.g. serious/severe effects); or

- indications of an effect for which the available evidence is inadequate for toxicological and/or risk characterisation; In such cases it may also be more appropriate to perform specific toxicological studies that are designed to investigate these effects (e.g. immunotoxicity, neurotoxicity); or

- particular concern regarding exposure (e.g. use in consumer products leading to exposure levels which are high relative to the dose levels at which toxicity to humans occurs).

A specialised study is likely to be more appropriate for an improved hazard characterisation and should be considered instead of a standard short-term rodent or sub-chronic toxicity test. An example of such an approach is given in Appendix R.7.5-1.

It should be pointed out that a failure to identify a NOAEL does not lead to a data gap in every case and should not be a default trigger for additional studies. If the data are sufficient for a robust hazard assessment or for C&L, the LOAEL may be used as the starting point for the CSA (see also Sections R.7.5.4.4 and R.7.5.5 and Chapter R.8 of the *Guidance on IR&CSA*).

R.7.5.6.3.3 1000 t/y or more (Annex X to the REACH Regulation)

There is no default testing requirement for repeated dose toxicity at this tonnage level beyond those recommended for the level 100 t/y or more (see above). However, in accordance with REACH Articles 40 and 41, if the frequency and duration of human exposure indicate that a

long-term study is appropriate and one of the following conditions is met, a long-term repeated toxicity test (≥12 months) may be proposed:

- serious or severe toxicity effects of particular concern were observed in the 28-day or 90-day study for which available evidence is inadequate for toxicological evaluation or risk characterisation; or
- effects shown in substances with clear relationship in molecular structure with the substance being studied were not detected in the 28-day or 90-day study; or
- the substance may have a dangerous property that cannot be detected in a 90-day study.

In addition, further studies must be proposed by the registrant or may be required by the Agency in accordance with REACH Articles 40 or 41, in case of:

- toxicity of particular concern (e.g. serious/severe effects); or
- indications of an effect for which the available evidence is inadequate for toxicological evaluation and/or risk characterisation; In such cases it may also be more appropriate to perform specific toxicological studies that are designed to investigate these effects (e.g. immunotoxicity, neurotoxicity); or
- particular concern regarding exposure (e.g. use in consumer products leading to exposure levels which are close to the dose levels at which toxicity is observed).

In some cases a specialised study might the most appropriate study in case an improved hazard characterisation is necessary and should be considered instead of a standard sub-chronic or chronic toxicity test. An example of such an approach given in Appendix R.7.5-1.

No further testing is required if the results of a sub-chronic rodent toxicity study (OECD TGs 408, 410, 411, 412, 413 / EU B.26, B.9, B.28, B.8, B.29) are adequate for a robust hazard characterisation and suitable for risk assessment and C&L (see Sections R.7.5.4.4 and R.7.5.5 for a detailed discussion of the criteria for a robust hazard characterisation).

Also, the testing requirements can be adapted if any of the rules according to Annex XI applies. For further details see Section R.7.5.6.3.5 on Annex XI below.

As there is no standard test requirement at this tonnage level, column 2 does not contain any waiving options.

R.7.5.6.3.4 Further considerations for studies that will be performed

In case a new study needs to be generated, the test has to be conducted in accordance with an appropriate test method and according to the principles of good laboratory practice. In addition, several considerations are required to ensure that the results will be appropriate for hazard identification. These are important for the selection of the most appropriate route of administration,

Selection of the most appropriate route of administration

A repeated dose toxicity study must be performed by either the oral, inhalation or dermal route. To decide on a specific route, it requires first to identify the appropriate routes. If more than one route is appropriate, a decision on the most appropriate route of administration is required.

Concerning repeated dose toxicity testing the oral route is the preferred one. However, depending on the physico-chemical properties of a substance, as well as on the most relevant route of human exposure, the dermal or the inhalation route could also be appropriate as specified in Annexes VIII and IX to the REACH Regulation.

It has to be noted that *in vivo* testing with corrosive substances at concentration levels causing corrosivity must be avoided.

Appropriateness of the dermal route of administration

Testing for repeated dose toxicity by the dermal route is appropriate if skin contact is likely and the physico-chemical properties suggest a potential for a significant rate of absorption through the skin. Testing for sub-acute toxicity (28 days) by the dermal route requires furthermore that inhalation of the substance is unlikely. Testing for sub-chronic toxicity (90-days) by the dermal route further requires that one of the following conditions is met:

- toxicity is observed in the acute dermal toxicity test at lower doses than in the oral toxicity test; or
- systemic effects or other evidence of absorption is observed in skin and/or eye irritation studies; or
- *in vitro* tests indicate significant dermal absorption; or
- significant dermal toxicity or dermal penetration is recognised for structurally-related substances.

If the substance is a severe irritant or corrosive, testing by the dermal route should be avoided unless it can be performed at doses that do not cause severe irriation or corrosion and provided that such doses are still toxicologically relevant for evaluating systemic toxicity and the outcome can be used in risk assessment.

A study by the dermal route might especially be required if route-to-route extrapolation is problematic, e.g. where a study with oral or inhalation administration does not allow reliable route-to-route extrapolation due to significant qualitative differences in metabolism in comparison with dermal exposure.

Appropriateness of the inhalation route of administration

Testing for repeated dose toxicity by the inhalation route is appropriate if exposure of humans *via* inhalation is likely, taking into account the vapour pressure of the substance and/or the possibility of exposure to aerosols, particles or droplets of an inhalable size.

Testing by the inhalation route is the default route for gases and the preferred route for liquids of high to very high vapour pressure at ambient temperature for which inhalation is usually the predominant route of human exposure.

For liquids of lower vapour pressure and for dusts (including nanomaterials), testing by the inhalation route is appropriate if human inhalation exposure is likely taking into account the possibility of exposure to aerosols, particles or droplets of an inhalable size. Further guidance on nanomaterials is available in Appendix R.7-1 *Recommendations for nanomaterials applicable to Chapter R.7a* of the *Guidance on IR&CSA.*

Selection of the most appropriate route of administration

In case more than one route of administration are appropriate, it is necessary to consider which is the ***most appropriate*** route of administration. This requires evaluating the advantages and disadvantages of all appropriate routes of administration.

Balancing of different routes of administration can include the following aspects:

- Preferred routes of administration, i.e.:
  - inhalation for gases and liquids of very high vapour pressure,
  - inhalatation, if effects may occur for which oral-to-inhalation extrapolation will not be appropriate; e.g.:
    - for nanomaterials
    - if there is some concern for systemic effects following inhalation exposure which might not be detected following oral administration[11]*
    - if there is some concern for local effects in the respiratory tract for which a qualitative assessment might not be sufficiently robust to demonstrate safe handling and use of the substance[12]**
  - oral for all other substances;
- Human exposure, e.g.:
  - route with presumed highest human exposure considering physico-chemical properties of the substance and its uses, with particular attention to exposure of professionals and/or consumers;
- Intrinsic properties/database, e.g.:
  - availability of route-specific information,
  - clarification of a concern for route-specific effect(s),
  - requirement of route-specific information to decide on the design of further test(s) like the extended one-generation reproduction toxicity study;
- Risk assessment, e.g.:
  - requirement of specific DNEL(s),
  - requirement of qualitative assessment,
  - application of risk management measures,
  - uncertainties,
  - proportionality;
- Feasibility.

Additional investigations

[11] Systemic effects that could occur following inhalation exposure might not be appropriately detected in a study with oral administration in case there are relevant route-specific toxicokinetic differences. For example, in case the substance undergoes a relevant first pass-effect in the gastro-intestinal tract or the liver after oral administration, the oral administration can be expected not to reflect the toxicity of the substance following inhalation exposure.

[12] A concern for local effects in the respiratory tract might be assumed *inter alia* for substances that are corrosive or irritating for the skin and/or eyes, substances that are hydrolysed/metabolised in the respiratory tract into reactive metabolites or insoluble inhalable dusts that accumulate in the lungs.

To adequately identify the hazard of a substance it might be necessary to perform additional investigations, which are either described as optional in the test methods or which are additional to the requirements of the test methods. Additional investigations can be triggered by existing information on the substance or on similar substances based on experimental or non-experimental sources of information that provide an indication for specific effects expected from the administration of the substance (i.e. in relation to neurotoxicity, immunotoxicity, endocrine disruption).

The possibility to explore several parameters within the design of the repeated dose toxicity could be considered (toxicokinetic data generation, micronucleus formation, neurotoxicity, immunotoxicity) taking into account potential limitations when modifying test protocols in order to investigate specific effects .

Investigations which are specifically required for nanomaterials (e.g. lung burden and Bronchoalveolar lavage (BAL) measurements) are indicated in Appendix R.7-1 *Recommendations for nanomaterials applicable to Chapter R.7a* of the *Guidance on IR&CSA*.

Toxicokinetics

The generation of toxicokinetic data should be considered in the light of the generation of other toxicity data (i.e. repeated dose toxicity) to assist in the estimation of internal exposure to the substance and/or its metabolites and the correlation of the effects observed with internal dose estimates. The latter is of particular importance for establishing the mode of action of the substance and whether administered doses caused saturation kinetics resulting in a non-linear dose-response. Such information is valuable for the derivation of assessment factors, route-to-route extrapolation and derivation of DNELs.

In addition, generation of toxicokinetic data (including metabolism characterisation) is considered essential for the application of read-across approaches as it contributes to the characterisation of common metabolic pathways as part of the similarity justification.

OECD TG 417 provides the protocol for the conduct of toxicokinetic studies either as stand-alone test or in combination with repeated dose toxicity studies.

Bronchoalveolar lavage (BAL) optional for inhalation studies

OECD TGs 412 and 413 for sub-acute and sub-chronic inhalation studies provide the option that, when there is evidence that the lower respiratory tract (i.e. the alveoli) is the primary site of deposition and retention, then bronchoalveolar lavage (BAL) may be the technique of choice to quantitatively analyse hypothesis-based dose-effect parameters focusing on alveolitis, pulmonary inflammation, and phospholipidosis. This allows for dose-response and time-course changes of alveolar injury to be suitably probed. BAL measurements generally complement the results from histopathology examinations but cannot replace them. Guidance on how to perform lung lavage can be found in OECD GD 39 (OECD, 2009). OECD TGs 412 and 413 are currently under revision and will include further guidance on BAL measurements.

Neurotoxicity and immunotoxicity

Information on the mode of action derived from the available data on the substance or data from structurally similar substances should be considered in the design of repeated dose toxicity tests. Such considerations can include inclusion of parameters to be measured in the test for elaborating a potential endocrine mode of action, neurotoxicity or immunotoxicity.

It should be noted that endpoints for detailed analysis of neurotoxicity and immunotoxicity are not examined in the standard 28-day and 90-day dermal or inhalation repeated dose toxicity studies. However it is stated in the updated OECD TG 413 (90-day inhalation study) that : "If neurotoxicity is expected or is observed in the course of the study, the study director may choose to include appropriate evaluations such as a functional observational battery (FOB) and measurement of motor activity."

Further Guidance on neurotoxicity is available in Appendix R.7.5-1.

If investigations regarding immunotoxicity need to be performed as part of the repeated dose toxicity test, these should be performed where relevant in a way that allows evaluation of the immunotoxicity potential (e.g. Repeated dose toxicity according to US EPA OPPTS 870.7800 – Health Effects Test Guidelines Immunotoxicity). Reviews of principles for immunotoxicity are available from WHO/IPCS publications and can be considered as additional guidance (WHO, 1996a; 1996b; 1999; 2007; 2012).

Endocrine mode of action

Regarding an endocrine mode of action, it should be noted that the oral 28-days study (OECD TG 407) gives more emphasis on the detection of endocrine-mediated effects than the oral 90-day study. The test protocol contains mandatory as well as optional endpoints for the detection of endocrine disruptors, providing information on estrogen- and androgen-mediated activity (agonistic and antagonistic), thyroid-related activity and steroidogenesis-related activity. Thereby it "allows certain endocrine mediated effects to be put into context with other toxicological effects."

Further guidance on Mode of action is available from the WHO/IPCS Framework on Mode of Action and Human Relevance (see http://www.who.int/ipcs/methods/harmonization/areas/cancer/en/).

In addition information from the OECD Adverse Outcome Pathway programme (see https://aopwiki.org/wiki/index.php/Main_Page) can provide insight into potential pathways relevant for the testing of a substance and the consideration of specific investigations that are likely to be relevant for a particular mode of action.

α2μ-globulin mediated nephropathy

If a substance leads to kidney effects in male but not in female rats, this may be indicative of an α2μ-globulin-mediated nephropathy. It is important to distinguish between a male-specific renal toxicity, which is not mediated by α2μ-globulin and which would be presumed relevant for human risk assessment, and α2μ-mediated nephropathy. Since humans do not have a functional α2μ-globulin gene, and this mode of action is considered not relevant to humans (IARC (1999), the involvement of α2μ-globulin in mediating the male rat-specific kidney effects is important for establishing the relevance of the kidney effects for risk assessment. To prove that the effects on the kidney are indeed mediated by α2μ-globulin, urinalysis (which is optional in the test methods) is required to investigate kidney function and full histopathological examination is required, including immuno-histochemical investigation to demonstrate the involvement of α2μ-globulin in the renal pathology (see for example Hamamura *et al.*, 2006).

Due to the extensive database on rats, this species is currently the preferred one to test substances that induce α2u-globulin-mediated nephropathy. However, in case α2u-globulin-mediated nephropathy is limiting the dose that can be applied, use of another species, e.g. the mouse, should be considered.

Additional parameters on reproductive toxicity

Repeated dose toxicity studies may be amended by including reproductive parameters like sperm parameters and/or oestrus cycles measurements. These examinations should be used to ensure the safe use of the substance. Performance of such investigations is at the discretion of a registrant.

Combination of studies

Considering animal welfare, it might be sensible to combinine a repeated dose toxicity study with a study that is required to fulfil a different information requirement. Combining studies lies in the responsibility of the registrants and requires careful consideration since a combination of studies also has drawbacks. It needs to be ensured that a combination of studies does not impair the validity and the results of the information of each individual study.

The combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD TG 422) is a comination of a sub-acute toxicity study and the screening study for reproductive/developmental toxicity. The advantages and disadvantages of this test are describend above (see Section R.7.5.4.1.2).

For combining a repeated dose toxicity study with an *in vivo* mammalian erythrocyte micronucleus test, specific considerations are provided in OECD TG 474.

Combining a sub-chronic toxicity study with the extended one-generation reproductive toxicity study could also be considered. Potential complications of such a combination might include selecting adequate dose levels to examine adequately both repeated dose toxicity and reproductive toxicity. In addition, interpretation of the results may be complicated due to differences in sensitivity between pregnant and non-pregnant animals (i.e. potential toxicokinetic differences), and an assessment of the general toxicity may be more difficult especially when serum and histopathological parameters are not evaluated at the same time in the study. Furthermore, the results of the sub-chronic toxicity study might be required before performing the extended one-generation reproductive toxicity study to decide on its study design.

R.7.5.6.3.5 REACH Annex XI adaptations of the standard testing regime for repeated dose toxicity

General guidance on the application of the Annex XI adaptations of information requirements is given in Chapter R.5 of the *Guidance on IR&CSA*. For repeated dose toxicity the following additional guidance applies:

Testing does not appear scientifically necessary

Some substances may be excluded from testing for repeated dose toxicity if it does not appear scientifically necessary (Annex XI Section 1). This might be the case for example if:

- a *Weight-of-Evidence* analysis demonstrates that the available information is sufficient for an adequate hazard characterisation and a CSA where the exposure to the substance is adequately controlled;
- a substance is not bioavailable *via* a specific route and possible local effects have been adequately characterised;
- the vapour pressure is sufficiently low that inhalational exposures are unlikely to be of significance, or if human exposure is limited to dusts or aerosols unlikely to be inhalable;
- for substances belonging to a group or a category of substances that have a common functionality and/or breakdown products or sufficient information for a qualitative and quantitative understanding of the toxicological properties, testing of all individual category members may not be necessary (Annex XI Section 1.5). The criteria for application of read-across for a category of substances and detailed guidance can be found in Sections R.4.3.2 and R.6.2 of the *Guidance on IR&CSA*.

Testing is technically not possible

There may also be cases where it is technically not possible to conduct a repeated dose toxicity test (Annex XI Section 2). This might be the case if:

- The substance ignites in air in ambient conditions;
- The substance undergoes immediate disintegration. In such a case the information requirements for the cleavage products should be assessed following an approach similar to that outlined in this document;
- The substance is corrosive in the dose range of interest for the study. Also, for reasons of animal welfare such studies should be avoided.

Substance-tailored exposure-driven testing

Exposure considerations may also lead to adaptation of the testing requirements (Annex XI Section 3). This might be the case if:

- Testing requirements may be adapted based on a substance-specific exposure assessment according to Annex XI Section 3. In this case testing for short-term repeated dose toxicity (Annex VIII, 8.6.1) may be waived at the 10-100 t/y tonnage level if relevant human exposure can be excluded (see Section R.7.5.4.3);
- Human exposure is limited at the tonnage level of 100 t/y or more (Annexes IX and X). The need for a sub-chronic study should be considered if the substance is only handled in industrial or commercial installations using closed systems and/or handled only as preparations at low concentrations.

R.7.5.7 References on repeated dose toxicity

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Appendix R.7.5-1 and 2 to Section R.7.5

Appendix R.7.5-1 Testing strategy for specific system/organ toxicity

Content of Appendix R.7.5-1

1. General aspects

For some specific system/organ effects the testing methods of the Annex to the EU Test Methods (TM) Regulation (Council Regulation (EC) No 440/2008) or of the OECD may not provide for adequate characterisation of the toxicity. There may be indications of such effects in the standard studies for systemic toxicity, or from SAR. For adequate characterisation of the toxicity and, hence, the risk to human health, it may be necessary to conduct studies using other published test methods, in-house methods or specially designed tests. Some references are given in Table R.7.5–3. Before initiating a study to investigate specific organ/system toxicity, it is important that the study design is presented to the Agency, in order that the need for (and scope/size of) studies using live animals can be particularly carefully considered.

Specific investigation of organ/systemic toxicity is to some extent undertaken as part of the repeated dose toxicity tests conducted according to test guidelines of the OECD and the Annex to the EU TM Regulation. Specific investigation (or further investigation) of any organ/system toxicity (e.g. immune, endocrine or nervous system) may sometimes be necessary and should be addressed on a case-by-case basis. As an example of a testing strategy the approach for neurotoxicity is given below.

2. Definition of neurotoxicity

Neurotoxicity is the induction by a chemical of adverse effects in the central or peripheral nervous system, or in sense organs. It is useful for the purpose of hazard and risk assessment to differentiate sense organ-specific effects from other effects which lie within the nervous system. A substance is considered *neurotoxic* if it induces a reproducible lesion in the nervous system or a reproducible pattern of neural dysfunction.

The starting point for the testing strategy are the REACH requirements specified in Annexes VIII, IX and X and detailed in Section R.7.5.6.3. Depending on the tonnage level, these requirements may trigger a 28-day and/or a 90-day test (e.g. OECD TGs 407, 408 / EU B.7, B.26). These protocols include a number of nervous system endpoints (e.g. clinical observations of motor and autonomous nervous system activity,

histopathology of nerve tissue), which should be regarded as the starting point for evaluation of a substance potential to cause neurotoxicity. It should be recognised that the standard 28-/90-day tests only measure some aspects of nervous system structure and function, e.g. Functional Observational Battery, while other aspects, e.g. learning and memory and sensory function is not or only superficially tested. SAR considerations may prompt the introduction of additional parameters to be tested in standard toxicity tests or the immediate request of studies such as delayed neurotoxicity (OECD TG 418 or 419 / EU B.37 or B.38; see below).

If there are no indications of neurotoxicity from available information i.e. adequately performed repeated dose toxicity tests, other testing systems (e.g. *in vitro*), non-testing systems ((Q)SAR and read-across) or human data, it will not be necessary to conduct any special tests for neurotoxicity.

The approach presented below is a hierarchical, stepwise strategy to investigate the potential neurotoxicity of a substance. It should be pointed out that the requirements outlined in steps 1 and 2 are met by the tonnage-based information requirements in Annexes VIII, IX and X to the REACH Regulation.

3. Structure-activity considerations

Structural alerts are only used as a positive indication of neurotoxic potential. Substance classes with an alert for neurotoxicity may include organic solvents (for chronic toxic encephalopathy), organophosphorus compounds (for delayed neurotoxicity) and carbamates (for cholinergic effects). Several estimation techniques are available, one of which is the rule-based DEREK (Deductive Estimation of Risk from Existing Knowledge) system. The rulebase comprises the following hazards and structural alerts: Organophosphate (for direct and indirect anticholinesterase activity), N-methyl or N,N-dimethyl carbamate (for direct anticholinesterase activity), gamma-diketones (for neurotoxicity).

4. Assessment of available information or results from initial testing

Signs of neurotoxicity in standard acute or repeated dose toxicity tests may be secondary to other systemic toxicity or to discomfort from physical effects such as a distended or blocked gastrointestinal tract. Nervous system effects seen at dose levels near or above those causing lethality should not be considered, in isolation, to be evidence of neurotoxicity. In acute toxicity studies where high doses are administered, clinical signs are often observed which are suggestive of effects on the nervous system (e.g. observations of lethargy, postural or behavioural changes), and a distinction should be made between specific and non-specific signs of neurotoxicity.

Neurotoxicity may be indicated by the following signs: morphological (structural) changes in the central or peripheral nervous system or in special sense organs; neurophysiological changes (e.g. electroencephalographic changes); behavioural (functional) changes; neurochemical changes (e.g. neurotransmitter levels).

A *Weight-of-Evidence* approach should be taken into account for the assessment of the neurotoxicity and the type, severity, number and reversibility of the effect should be considered. A consistent pattern of neurotoxic findings rather than a single or a few unrelated effects should be taken as persuasive evidence of neurotoxicity.

It is important to ascertain whether the nervous system is the primary target organ. The reversibility of neurotoxic effects should also be considered. The potential for such effects to occur in exposed humans (i.e. the exposure pattern and estimated level of exposure are *acute*) should be considered in the risk characterisation. Reversible effects may be of high concern depending on the severity and nature of effect. In this context it should be

kept in mind that effects observed in experimental animals that appear harmless might be of high concern in humans depending on the setting in which they occur (e.g. sleepiness in itself may not be harmful, but in relation to operation of machinery it is an effect of high concern). Furthermore the possibility that a permanent lesion has occurred cannot be excluded, even if the overt effect is transient. The nervous system possesses reserve capacity, which may compensate for the damage, but the resulting reduction in the reserve capacity should be regarded as an adverse effect. Irreversible neurotoxic effects are of high concern and usually involve structural changes, though, at least in humans, lasting functional effects (e.g. depression, involuntary motor tremor) are suspected to occur as a result of neurotoxicant exposure, apparently without morphological abnormalities.

For the evaluation of organophosphate pesticides, the WHO/FAO Joint Meeting of Experts on Pesticide Residues (JMPR) has published recommendations on "Interpretation of Cholinesterase Inhibition" (FAO, 1998; 1999). The applicability of these recommendations, outlined below, could also be extended to other substances that inhibit cholinesterase. It should be pointed out that for substances that may have a structural alert for cholinesterase inhibition, the measurement of acetylcholinesterase activity as recommended by JMPR can be included in the list of parameters for the standard 28- or 90-day testing protocols required by REACH, irrespective of the route of exposure.

5. Recommendations from the WHO/FAO Joint Meeting of Experts on Pesticide Residues (JMPR)

The inhibition of brain acetylcholinesterase activity and clinical signs are considered to be the primary endpoints of concern in toxicological studies on compounds that inhibit acetylcholinesterases. Inhibition of erythrocyte acetylcholinesterase is also considered to be an adverse effect, insofar as it is used as a surrogate for brain and peripheral nerve acetylcholinesterase inhibition, when data on the brain enzyme are not available. The use of erythrocyte acetylcholinesterase inhibition as a surrogate for peripheral effects is justified for acute exposures resulting in greater acetylcholinesterase inhibition in erythrocytes than in the brain. However, reliance on inhibition of erythrocytic enzyme in studies of repeated doses might result in an overestimate of inhibition on peripheral tissues, because of the lower rate of resynthesis of the enzyme in erythrocytes than in the nervous system. Plasma acetylcholinesterase inhibition is considered not relevant. Regarding brain and erythrocyte acetylcholinesterase inhibition, the experts defined that statistically significant inhibition by 20% or more represents a clear toxicological effect and any decision to dismiss such findings should be justified. JMPR also agreed on the convention that statistically significant inhibition of less than 20% or statistically insignificant inhibition above 20% indicate that a more detailed analysis of the data should be undertaken. The toxicological significance of these findings should be determined on a case-by-case basis. One of the aspects to consider is the dose-response characteristic.

6. Further neurotoxicity testing

If the data acquired from the standard systemic toxicity tests required by REACH provide indications of neurotoxicity which are not adequate for a hazard assessment, risk characterisation or C&L, the nature of further investigation will need to be considered. If a 90-day study is triggered to meet the requirements of Annex IX to the REACH Regulation following a standard 28-day study, a number of endpoints assessing the nervous system endpoints should be included, irrespective of the administration route. In some cases, it may be necessary to conduct a specific study such as a neurotoxicity test using the OECD TG 424 with possible inclusion of a satellite group for assessment of reversibility of effects. The OECD TG 424 is intended for confirmation or further

characterisation of potential neurotoxicity identified in previous studies. The OECD guideline allows for a flexible approach, in which the number of simple endpoints which duplicate those already examined during standard testing may be minimised, and where more effort is put into in-depth investigation of more specific endpoints by inclusion of more specialised tests. Adjustment of dose levels to avoid confounding by general toxicity should be considered.

If data from standard toxicity studies are clearly indicative of specific neurotoxicity, e.g. neurotoxicity occurring at lower dose levels than systemic toxicity, further specific neurotoxicity testing is required to confirm and extend the findings from the general toxicity studies and to establish an NOAEL for neurotoxicity. Again, the neurotoxicity test according to OECD TG 424 is considered appropriate for this situation.

Certain substances and/or certain effects are best investigated in particular species. Pyridine derivatives are neurotoxic to humans and primates but not to rats. Among other neurotoxic compounds, organophosphorus compounds are a group with known delayed neurotoxic properties, which need to be assessed in a specified test for delayed neurotoxicity, to be performed preferentially in the adult laying hen according to EU B.37 or OECD TG 418 (Delayed neurotoxicity of organophosphorus substances following acute exposure) and B.38 or OECD TG 419 (Delayed neurotoxicity of organophosphorus substances: 28-day repeated dose study). Such studies are specifically required for biocidal substances of similar or related structures to those capable of inducing delayed neurotoxicity. If anticholineesterase activity is detected, a test for response to reactivating agent may be required.

Standard exposure conditions may not always be adequate for neurotoxicity studies. The duration of exposure needed to induce specific neurotoxic effects in an animal experiment will depend on the underlying mechanism of action. Short-term peak exposures can be important for certain types of substance/effect. When the test compound is administered as a bolus *via* the intravenous, subcutaneous or oral route it is essential to determine the time-effect course, and to perform measurements of neurotoxicity parameters preferentially at the time of peak effect.

For example, the neurotoxicity associated with short-term exposure to some volatile organic solvents has largely been identified following human exposure, particularly occupational exposure. Acute inhalation studies, using protocols designed to detect the expected effects, are ideal for such substances/effects. For some neurotoxic substances a long exposure period is necessary to elicit neurotoxicity.

The most appropriate methods for further investigation of neurotoxicity should be determined on a case-by-case basis, guided by the effects seen in the standard systemic toxicity tests and/or from SAR-based predictions. Extensive coverage of methods that may be used can be found in the documents issued by the OECD (2004), WHO (1986) and ECETOC (1992), and some methods are summarised in Table R.7.5–3.

Table R.7.5–3 Methods for investigation of neurotoxicity

Effect	Methods available	References*
Morphological changes	Neuropathology. Gross anatomical techniques. Immunocytochemistry. Special Stains	Krinke, 1989; Odonoghue, 1989; Mattson *et al.*, 1990
Physiological changes	Electrophysiology (e.g. nerve conduction velocity (NCV), Electroencephalogram (EEG), evoked potentials	Fox *et al.*, 1982; Rebert, 1983; Mattson and Albee, 1988
Behavioural changes	Functional observations. Sensory function tests. Motor function tests (e.g. locomotor activity). Cognitive function tests	Robbins, 1997; Tilson *et al.*, 1980; Cabe and Eckerman, 1982; Pryor *et al.*, 1983 Moser and MacPhail, 1990; Moser 1995
Biochemical changes	Neurotoransmitter analysis. Enzyme/protein activity. Measures of cell integrity.	Dewar and Moffet, 1979; Damstra and Bondy, 1982; Cooper *et al.*, 1986; Costa, 1998.

*Given in full in ECETOC (1992), WHO (1986) or Mitchell (1982)

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Appendix R.7.5-2 (Q)SARs for the prediction of repeated dose toxicity

A number of *in silico* tools are available for the prediction of repeated dose toxicity.

As already stated in the main text of this Section, the use of these tools should be mainly for obtaining screening and mechanistic information. Some of them are presented in Table R.7.5–4 below. A more exhaustive review of the available databases, literature and *in silico* models is given in a JRC report from Lapenna *et al.*, 2010.

Table R.7.5–4 *in silico* tools for the prediction of repeated dose toxicity

Tool	Model/module	Description
QSAR Toolbox (Free) http://www.qsartoolbox.org/	Profilers and databases	Co-developed by ECHA and OECD, the QSAR Toolbox includes specific profilers (e.g. Repeated dose HESS) and databases (e.g. Fraunhofer ITEM) for repeated dose toxicity. These modules facilitate the selection of analogues with repeated dose toxicity experimental data for filling data gaps via read-across or trend-analysis.
ADMET Predictor (Simulation Plus) (Commercial) http://www.simulations-plus.com/Products.aspx?pID=13&mID=27	Toxicity	The toxicity module in ADMET Predictor includes a series of models for various organ toxicities (e.g. cardiac, liver).
Derek Nexus (Lhasa) (Commercial) https://www.lhasalimited.org/products/derek-nexus.htm	Models for organ toxicity	Derek Nexus includes several specific organ toxicity models related to repeated dose toxicity (e.g. liver).
Discovery Studio (BIOVIA) (Commercial) http://accelrys.com/products/collaborative-science/biovia-discovery-studio/qsar-admet-and-predictive-toxicology.html	TOPKAT	TOPKAT (TOxicity Prediction by Komputer Assisted Technology) includes a model for Rat chronic LOAEL.
Leadscope (Commercial) http://www.leadscope.com/index.php	Various organs adverse effects statistical models	Leadscope includes several specific organ toxicity models related to repeated dose toxicity (e.g. hepatobiliary tract).

Reference

Lapenna S, Fuart-Gatnik M and Worth A (2010) Review of QSAR models and software tools for predicting acute and chronic systemic toxicity. JRC report EUR 24639 EN. Publications Office of the European Union. Available at: http://publications.jrc.ec.europa.eu/repository/handle/JRC61930