Category approaches, Read-across, (Q)SAR

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TABLE OF CONTENTS

SUMMARY **1**

1. INTRODUCTION **3**

1.1 Terms of reference *4*

1.2 Scope *5*

1.3 Roadmap of the report *6*

2. DEFINITIONS FOR NON-TESTING APPROACHES **8**

2.1 Data gap filling *9*

2.1.1 Read-across 9

2.1.2 Trend analysis and computational methods based on internal models 13

2.1.3 External (Q)SAR models and expert systems 14

3. RESOURCES FOR NON-TESTING APPROACHES **15**

3.1 Regulatory guidance *15*

3.1.1 ECHA guidance 15

3.1.2 OECD guidance 17

3.1.3 USA EPA High Production Volume (HPV) Challenge Program 20

3.1.4 Canada Substance Groupings Initiative 21

3.1.5 Japan HPV Challenge Program 21

3.1.6 China New Chemical Substance Notification 22

3.1.7 TSCA New Chemicals Program (NCP) Chemical Categories 22

3.2 Scientific literature *22*

3.3 Software resources *24*

3.3.1 (Q)SAR/Expert systems 25

3.3.2 Prediction of metabolism 32

3.3.3 Grouping tools 34

4. END APPLICATIONS – FITNESS FOR PURPOSE **38**

4.1 Top-down: screening/prioritisation *38*

4.2 Bottom-up: regulatory purposes *40*

4.3 Top-down/Bottom-up: special class considerations *41*

5. CONSIDERATIONS FOR THE USE OF NON-TESTING APPROACHES **44**

6. SYSTEMATIC WORKFLOW **47**

6.1 Analogue identification *47*

6.2 Rationale for grouping: analogue evaluation *52*
6.2.1 Common functional group(s) 53
6.2.2 Incremental and constant change across the category 54
6.2.3 Common constituents or chemical classes 55
6.2.4 Other considerations for analogue evaluation 55
6.2.5 The metabolic pathway justification 56
6.2.6 Read-across considerations for metal compounds 59
6.3 Considerations of impurities/purities *59*
6.4 List of endpoints covered *60*
6.5 Endpoint by endpoint justification *64*
6.5.1 Physicochemical parameters 64
6.5.2 Aquatic toxicity 65
6.5.3 Biodegradation 66
6.5.4 Bioaccumulation 68
6.5.5 Acute mammalian toxicity 71
6.5.6 Irritation 73
6.5.7 Skin sensitisation 74
6.5.8 Respiratory sensitisation 78
6.5.9 Mutagenicity 78
6.5.10 Repeated-dose toxicity 80
6.5.11 Reproductive and developmental toxicity 83
6.6 Classification and labelling comparison *87*

7. RECOMMENDATIONS **88**

8. RESEARCH NEEDS/ACTIVITIES **90**

ABBREVIATIONS **93**

BIBLIOGRAPHY **97**

APPENDIX A: WORKFLOWS **112**

APPENDIX B: Case Studies **115**
B.1. Cycla-esters *115*
B.2. Alkyldimethylamines (ADA) *129*
B.3. Montan wax derived chemicals *132*
B.4. Glycol ethers E series category *135*
B.5. Lower alkyl methacrylate esters *160*
B.6. Molybdenum compounds *174*

MEMBERS OF THE TASK FORCE **181**

MEMBERS OF THE SCIENTIFIC COMMITTEE **182**

SUMMARY

Considerable practical experience has been gained in applying non-testing approaches for regulatory purposes, most recently driven by the demands of the REACH legislation (EC, 2006). This ECETOC Task Force was convened to summarise guidance and tools available, to review their practical utility and to consider what technical recommendations and learnings could be shared more widely to refine and inform on the current use of read-across. A number of case studies were formulated and the generic insights of developing, evaluating, justifying and documenting read-across approaches were extracted as far as possible. Sharing this experience aims to inform users about the pitfalls and challenges associated with read-across approaches as well as about feasible practical strategies available to develop appropriate scientific justifications. The report is intended to lay down the foundations of robust yet practical read-across approaches whilst encouraging consistent application across industry.

Currently read-across strategies for REACH have tended to rely on the so-called 'analogue approach' as opposed to a 'category approach', in addition to (quantitative) structure activity relationship [(Q)SAR] approaches. (Q)SAR approaches have been extensively relied upon to address data gaps for physicochemical properties such as log K_{ow}, environmental fate parameters such as biodegradation, hydrolysis, bioaccumulation potential and ecotoxicity endpoints such as acute aquatic toxicity in the standard species (fish, *daphnia* and *algae*). For the aforementioned properties, such (Q)SAR approaches have been used as direct replacements to experimental testing. There are many expert systems available to facilitate these assessments including the Organisation for Economic Cooperation and Development (OECD) (Q)SAR Toolbox or the United States Environmental Protection Agency (US EPA) EPISUITE programme. For mammalian endpoints, (Q)SAR have been applied less frequently with the exception of endpoints such as Ames mutagenicity and, to a lesser extent, skin sensitisation where the mechanisms of action are relatively well understood and where the underlying data are more readily available. Read-across approaches have been attempted as a means to address data gaps for longer-term effects such as 90-day repeated-dose toxicity studies or reproductive/developmental effects. For such complex endpoints, (Q)SAR have been applied, but their role has been as supporting information as a means to highlight potential chemical modes of action or to offer indications for similarity in effect. For such endpoints in particular, information on likely transformation products and the rate of formation of these products as derived from experimental studies are strongly recommended to substantiate the overall read-across justification. Whilst toxicokinetic information is not a requirement under REACH *per se*, such information is viewed as key to help rationalise certain read-across approaches, particularly for endpoints like reproductive/developmental effects where current (Q)SAR approaches are still in early development.

Absence of toxicity is a particular challenge to justify. Despite provisions in REACH calling for the use of read-across and (Q)SAR for both the absence and presence of toxicity (see Annex XI in EC, 2006), the justification of 'absence' is not to be underestimated. In many cases, toxicokinetic information or physiologically-based pharmacokinetic (PBPK) modelling is considered desirable to provide valuable supporting evidence.

Read-across approaches for longer-term effects should ideally be structured to present an overall 'weight of evidence' (WoE) argument (SCENIHR, 2012). A justification needs to rely on several lines of corroborating evidence whether it be consistent metabolic profiles, similarity in effects at shorter exposures, (Q)SAR estimates or other supporting analogues with experimental data that are not necessarily part of the main

category/analogue approach. In the latter case, tools such as Toxmatch, Leadscope or the OECD (Q)SAR Toolbox may prove helpful to identify related analogues (Patlewicz et al, 2011).

It is recommended that justifications are structured using a template such as the category/analogue reporting format (CRF/ARF) as outlined in OECD (OECD, 2007a) and REACH guidance documents (ECHA REACH TGD). These templates are an effective means of structuring the arguments on an endpoint per endpoint basis as well as presenting an overall data matrix for the analogue or category member under evaluation. A justification is strengthened by the presentation of an explicit data matrix which demonstrates a consistent profile for the members of the category or analogues under consideration. Presenting data for the source analogues for the endpoint that is proposed to be read across alone may not be sufficient.

By default, read-across is considered to be associated with additional uncertainty due to the fact that information on a target substance is being inferred from that available on a source substance(s). Whilst assessment factors can be a route by which uncertainty is addressed, these should be used on a case by case basis and driven by the confidence associated with the underlying similarity hypothesis as well as the quality of the study data forming part of the supporting WoE information.

In the future, less 'classical' toxicity data will be anticipated for each individual analogue member, and rather more 'omics data (van Ravenzwaay et al, 2012). Thus, there will likely be a commensurate shift towards deriving larger categories as contrasted with analogue approaches. This should facilitate analysis of trends, although the data gap-filling approaches will likely be contingent on the application of non-standard, alternative toxicity testing data including that from high throughput/high content technologies. Whilst this will be a challenge in interpretation, it does present a cost-efficient means of generating data in a relatively short time frame.

Guidance and experience will continually evolve as Tox21 (See Chapter 1 for further details) activities progress. US Environmental Protection Agency's ToxCast is one such example (Judson et al, 2010) and the OECD's adverse outcome pathway (AOP) work programme is another (OECD, 2011). Both will have an impact on the development of read-across justifications. AOPs may in the future have the potential to provide the conceptual framework for how to utilise alternative data in the appropriate biological context as well as the chemical anchor by way of molecular initiating events (MIEs). Datasets such as those generated in ToxCast and related programmes may ultimately help to formulate practical strategies to quantify AOPs. OECD's grouping guidance, which is currently under revision, discusses AOPs as a means towards developing new categories and read-across that are more mechanistically based (OECD, 2011). AOPs will also be implemented in some fashion in the OECD (Q)SAR Toolbox to extend the scope of its functionality. It is anticipated that regulatory agencies will start to consider these approaches. Indeed, the US EPA have alluded to a shift in the development of their chemical categories from those that are purely based on structure and physicochemical properties to ones that rely on the concepts of AOP information to inform their development and evaluation (Seed, 2012).

This report presents a snapshot of current practices and highlights possible future needs and opportunities. Read-across is clearly evolving and the challenge will be to keep pace and drive the scientific development and evaluation of these approaches including the critical assessment of its limitations.

1. INTRODUCTION

(Quantitative) Structure-activity relationships ((Q)SAR), chemical categories and read-across form a continuum of non-testing approaches based on the hypothesis that chemical similarity is related to biological activity, i.e. similar chemicals should exhibit similar biological profiles. Chemical similarity is not, however, limited to structural similarity but should consider the factors that drive a given toxicity and how these can be linked back to chemical properties or features e.g. reactivity. The application of these approaches has been largely routine as part of research and development pipelines within many companies to rank substances of interest based on their predicted toxicity profile or to inform targeted testing as part of risk assessments. In the regulatory arena, non-testing approaches have been used for screening purposes; notable examples include those in use by the US EPA as part of their review of Pre-Manufacture Notices (PMN) filed under the remit of the Toxic Substances Control Act (TSCA) of 1976 as well as the assessment of the Canadian Domestic Substances List (DSL). On an international level, OECD has also played a significant role in promoting the use of category-based approaches for the assessment of High Production Volume (HPV) chemicals.

During about the last 10 years, there has been considerable change in the regulatory landscape and a call to exploit alternative approaches to fulfil more than just screening or prioritisation purposes. This shift was in part initiated within the EU in response to the Cosmetics Directive (EC, 2003) calling for a ban on animal testing with certain deadlines for specific endpoints. Following the publication of the White Paper in 2001 which outlined the REACH regulation (EC, 2001), many activities to promote the application and interpretation of non-testing approaches were also initiated. The ICCA-LRI Setubal workshop was one such activity which brought together a wide international stakeholder group to formulate guiding principles for the development and application of (Q)SAR (Cronin et al, 2003; Cefic-LRI, 2002). These were subsequently taken up by the OECD and adopted formally as the OECD validation principles for (Q)SAR (OECD, 2004). Further information is provided in Chapter 3 of this report. OECD Guidance was then drafted (OECD, 2007b) to characterise each of the principles based on the preliminary guidance developed by the Joint Research Centre's European Chemicals Bureau (Worth et al, 2005). During the development of the REACH technical guidance document, guidance was also written to assist in the development and application of (Q)SAR. This includes specific formats to facilitate documentation and justification of (Q)SAR models and the predictions they generate. The validation principles formed a framework underpinning both these reporting formats. For chemical categories, the respective OECD HPV manual formed the starting point for the development of the corresponding REACH guidance (ECHA, http://www.echa.europa.eu 2012) as well as an updated OECD guidance (OECD, 2007a). The REACH and updated OECD guidance are to all intents and purposes identical. The aim was to develop revised regulatory guidance that would prove to be more practical in terms of outlining the strategies for developing, justifying and documenting chemical categories. Workflows were developed to illustrate the different steps that could be taken, and reporting formats were described to outline the key characteristics that needed to be discussed as part of a category approach. Indeed this guidance proposes stepwise approaches for analogue and category read-across. The steps include: (1) identifying potential analogues, (2) gathering data on these potential analogues, (3) evaluating the adequacy of data for each potential analogues, (4) constructing a matrix with available data for the target and analogue(s), (5) assessing the adequacy of the analogue(s) to fill the data gap, and (6) documenting the entire process. The REACH/OECD guidance also indicates the importance of comparing the physicochemical properties of the analogue and target chemicals as well as assessing the likely toxicokinetics of the

substances, including the possibility that divergent metabolic pathways could be an important consideration. Whilst the guidance presents a step change in terms of defining (Q)SAR principles such as applicability domains, it does not provide specific details for assessing whether an analogue is appropriate for filling a data gap nor the extent of detail that is required to document an approach nor what might be guiding principles to evaluate 'fitness for purpose'. The guidance also provided no specific examples on how to develop category approaches (Wu et al, 2010).

Since the development of the REACH/OECD guidance, there has been a wealth of effort focused on the OECD (Q)SAR Toolbox (OECD, 2009a), a project currently sponsored by the European Chemicals Agency (ECHA) for the development, justification and documentation of endpoint-specific chemical categories. This tool provides some of the features to help evaluate analogue suitability and relevance. A guidance document is available to assist the development of categories. More specific information is provided in Chapter 3 together with additional information on other related tools.

Following publication of the report Toxicity Testing in the 21st Century: A Vision and a Strategy (NRC, 2007), there has been a move towards redesigning the paradigm for toxicological risk assessment. In the future, the use of high-throughput/high-content assays (HT/HC) could be part of the suite of next generation assays that could be potentially applied for screening purposes for risk assessment. The ToxCast programme from the US EPA is an example of such technology (Judson et al, 2010). Another role such assays could possibly play is as anchor data for chemical categories or quantifying dose-responses as part of an adverse outcome pathway (AOP) or mode of action (MOA) framework (Patlewicz et al, 2012). The ECETOC Technical Report 109 'High information content technologies in support of read-across in chemical risk assessment' highlighted how HT/HC information could be used to substantiate a category or analogue approach (ECETOC, 2010). The OECD held a workshop to discuss and outline activities to start the process of incorporating AOP information to help develop mechanistically relevant categories (OECD, 2011; also referenced in Chapter 3 of this report). One of the recommendations highlighted during the course of the workshop was that a library of AOPs should be developed. Further work under OECD will be carried out from 2013 onwards (Diderich, 2011).

1.1 Terms of reference

Some non-testing approaches are well-established and widely used for the assessment of human and environmental safety of chemicals. These approaches are acceptable, with limitations, in preparing dossiers for REACH, and other regulatory programmes. Whilst general guidance and many models and tools are available to assist in the development of categories and read-across, practical guidance is still missing. Areas where such additional practical guidance would be helpful are guiding principles for formulating hypotheses and for evaluating analogues, how to characterise uncertainties associated with read-across approaches and how these may differ depending on the endpoint under consideration, as well as on the extent and depth of read-across documentation depending on the intended application e.g. company specific product stewardship, regulatory classification and labelling or hazard characterisation.

Proposing validation principles akin to those derived for (Q)SAR may be a useful component to help assess the adequacy of these types of end applications. A report describing such guiding principles and offering

recommended practices would be useful in supporting risk characterisation and prioritisation activities across all sectors.

A Task Force was established to prepare such a report. The terms of reference were agreed as follows:

1. Collate published literature and regulatory guidance describing/cataloguing the development of chemical categories and use of read-across and (Q)SAR in human health and environmental risk assessment.
2. Develop recommended practices for identifying chemical categories and analogues to meet scientific rigour, including hazard identification and risk characterisation, and classification and labelling. Develop a proposal how to use structure activity relationship (SAR) in higher tier testing strategies.
3. Determine endpoint-specific methods (e.g. (Q)SAR, rulebase models) and their limitations in terms of their predictive value (e.g. with respect to applicability domain). If possible clarify minimum requirements to apply a category approach.

This report is structured to address these terms of reference.

1.2 Scope

Given the wealth of regulatory guidance and peer reviewed literature on (Q)SAR, chemical categories and read-across, this report aims to collate the key references and resources available. It does not review each of the citations given since these have been the subject of other publications. Any recent information or pertinent updates from ECHA, e.g. Read-Across Assessment Framework (RAAF), are discussed for completeness since these are unlikely to be referenced elsewhere.

Recommended practices for identifying and evaluating analogues as well as proposed 'validation' principles are drawn largely from a selection of analogue/category case studies that are cited in the Appendices.

Whilst tools and approaches are mostly tailored to address categories/analogues comprising discrete organic chemicals, the principles and recommendations are generally applicable to all types of chemicals. Special considerations where merited are discussed for metals, metal containing compounds and UVCBs (Substances of Unknown or Variable Composition, Complex reaction products or Biological materials). For the latter, extensive work is on-going and the focus is to cite pertinent documentation. As read-across approaches for polymers and nanomaterials are under development, these are out of the scope of this document.

This report is targeted towards industry risk assessors and eco-/toxicologists that have an awareness and interest in grouping approaches including read-across. ECHA scientists and those from other regulatory bodies may find this report informative since it aims to explain in as systematic a way as possible, the procedures and practices that industry intends to follow for grouping approaches as well as its own guiding principles and recommendations.

1.3 Roadmap of the report

This ECETOC Task Force report provides practical guidance for anyone interested in developing or evaluating read-across justifications as part of analogue or category approaches. Since the report itself is sizeable, a roadmap is oulined to help guide the reader.

Chapter 1 provides a general introduction and background to frame read-across as a topic and to present the scope of the report itself.

Chapter 2 provides working definitions of many of the terms used in the field of non-testing approaches. Since these are routinely used in the remainder of the text, the aim is to provide clear definitions upfront that can be referenced as required.

Chapter 3 provides a summary of available resources – what regulatory guidance is already published, what scientific publications may be insightful and what software tools may be of potential consideration to facilitate a grouping exercise.

Chapter 4 highlights how the context of use can impact and dictate the overall grouping strategy. Being mindful of what question is being addressed can frame the tactical approach of how the grouping should be tackled or, depending on what information is available or the types of substances being evaluated, what practical grouping strategies are even feasible. There is no one size fits all as far as how an analogue or category approach may be undertaken. It is context specific.

Chapter 5 outlines some pertinent considerations to bear in mind before embarking on a grouping strategy.

Chapter 6 details the practical steps involved in a grouping exercise. It outlines the mechanics of identifying analogues and evaluating their suitability, through to formulating a hypothesis and justifying the similarity on an endpoint per endpoint basis. The chapter is structured to mirror the considerations outlined in the reporting formats cited in the regulatory guidance available under REACH and OECD.

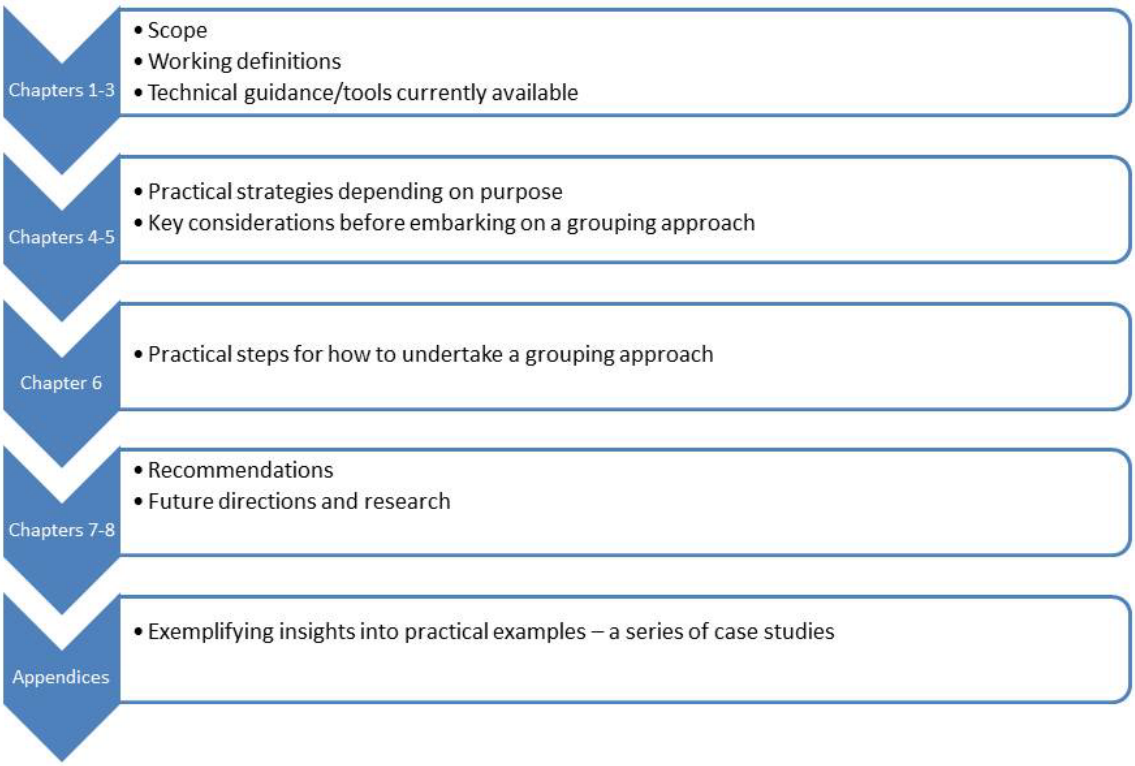
Chapter 7 and 8 highlight interim or longer-term efforts that could be undertaken to refine and improve the manner in which read-across is performed.

Finally, the Appendices provide some case studies which exemplify the strategies and insights described in the main body of the report.

For the reader who requires a brief synopsis of the available guidance/resources, Chapter 3 is most informative. For an account manager or risk assessor who needs to familiarise themselves with the relative risks and benefits of undertaking a grouping strategy, Chapter 5 is key.

For the reader with strong technical expertise in read-across but who seeks guiding principles of how to justify a read-across and what the final document may look like, Chapter 6 and the associated Appendices are critical.

A graphical summary is provided below:



2. DEFINITIONS FOR NON-TESTING APPROACHES

In this report, the terms 'category approach' and 'analogue approach' are used to describe techniques for grouping chemicals, whilst the term 'read-across' is reserved for a technique of filling data gaps in either approach. Definitions are taken from the REACH technical guidance (ECHA REACH TGD) which is discussed in Chapter 3 unless otherwise stated. Analogue approach is often used when the grouping is based on a very limited number of chemicals, typically two substances. A chemical category describes a group of chemicals whose physicochemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity (or other similarity characteristic). The rationale for forming the category may be based on a range of different similarity characteristics. The following is not intended to be exhaustive but frames several of the similarity contexts that could be used:

- On account of a common functional group (e.g. aldehyde, epoxide, ester, specific metal ion).
- Common constituents or chemical classes, similar carbon range numbers; an incremental and constant change across the category (e.g. a chain-length category).
- Likelihood of common precursors and/or breakdown products, via physical or biological processes, which result in structurally similar chemicals (e.g. the metabolic pathway approach of examining related chemicals such as ester/acid/salt).

These contexts are significant in framing the type of rationale and supporting justification needed to substantiate the overall category approach. This is discussed in more detail in Chapter 6. The expectation is that these will be utilised by ECHA as part of their RAAF Tier 2 evaluation.

The assessment of chemicals by using a category approach differs from the approach of assessing them on an individual basis, since the effects of the individual chemicals within a category are assessed on the basis of the evaluation of the category as a whole, rather than based on measured data for any one particular substance alone. This may have downstream consequences in terms of classification and labelling and/or risk characterisation.

The category approach is, by its very nature, a weight of evidence approach (WoE), since it typically integrates both estimated and experimental data, and involves expert judgment. A WoE can be categorised as either qualitative (i.e. a determination of what is reasonable in view of all available information) or quantitative (i.e. a process by which conclusions are reached through assignment of numerical weights to scientific evidence) (Nichols et al, 2009). The category approach also provides a means of strategic testing. The biggest challenge in this approach lies in defining the category itself (its underlying rationale/mechanistic basis) and in particular its boundaries.

The wider category approach is perceived to be more robust than simple analogue approaches, which are limited to small numbers of substances. As the number of possible chemicals being grouped into a category increases, the potential for developing hypotheses for specific endpoints and making generalisations about the trends within the category also increases, and hence improves the robustness of the evaluation. For analogue approaches, substantiating the hypothesis for a specific endpoint also relies upon a WoE approach. Whilst trends cannot be routinely established given only two substances *per se*, several lines of

corroborating evidence may be collected to support the similarity hypothesis underpinning the proposed read-across. Information regarding the metabolic fate, consistent predictions from relevant (Q)SAR models, comparable physicochemical properties as well as commonality in reactivity profiles should help to provide an overall profile of the likely hazard under consideration and demonstrate consistency between source and target substances. Workflows describing the considerations of the WoE are provided in Appendix A.

When applying the category approach, the robustness of the overall category is assessed; rather than the reliability for an individual substance, since in some cases, individual substances may display exceptional behaviour. Thus, the adequacy (relevance and reliability) of the approach needs to be assessed for individual substances of interest.

2.1 Data gap filling

Whenever a category is formed, data gaps may be filled in using read-across (qualitative or quantitative), trend analysis (local (Q)SAR) and external (Q)SAR models and expert systems. All four techniques have a lot in common, and are often interrelated. The techniques are described briefly, with most emphasis on read-across.

2.1.1 Read-across

Endpoint information for one chemical is used to predict the same endpoint for another chemical, which is considered to be similar in some way (usually on the basis of structural similarity or same mode of action or other properties). Read-across can be used to fill data gaps in the context of both the analogue approach and the wider category approach. In principle, read-across can be applied to characterise physicochemical properties, environmental fate, human health effects and ecotoxicity. For any of these endpoints, read-across may be performed in a qualitative or quantitative manner.

Read-across possibilities

Read-across can be performed by following any of the four approaches listed below:

- One-to-one (one analogue used to make an estimation for a single chemical);
- many-to-one (two or more analogues used to make an estimation for a single chemical);
- one-to-many (one analogue used to make estimations for two or more chemicals);
- many-to-many (two or more analogues used to make estimations for two or more chemicals).

Qualitative read-across

Qualitative read-across is similar to the use of a SAR where a SAR is a qualitative association between a chemical substructure and the potential of a chemical containing the substructure to exhibit a certain biological effect. The process involves:

- The identification of a chemical substructure or mode or mechanism of action that is common to two substances (which are considered to be analogues); and
- the assumption that the presence (or absence) of a property/activity for a substance can be inferred from the presence (or absence) of the same property/activity for the analogous substance.

The main application of qualitative read-across is in hazard identification.

Quantitative read-across

Quantitative read-across involves:

- The identification of a chemical substructure or mode or mechanism of action that is common to two substances (which are considered to be analogues); and
- the assumption that the known value of a property for one substance can be used to give a quantitative estimation of the unknown value of the same property for another substance.

In both cases, expert judgement is needed and justification should be provided.

Especially with respect to a quantitative read-across the following approaches can be utilised to fill in the data gaps:

- Use the endpoint value of a source chemical, e.g. the closest analogue in a (sub)category.
- Use an internal (Q)SAR model to scale the available experimental results from two or more source chemicals.
- Process the endpoint values from two or more source chemicals (e.g. averaging, taking the most representative value).
- Take the most conservative value of the closest analogues.

When to carry out read-across

Read-across can be carried out in order to fill a data gap for a specific endpoint, to help to derive a more realistic predicted no effect concentration/derived no effect level (PNEC/DNEL) or to identify/flag a concern that may be better addressed by further testing. In principle, read-across can be applied for any property or endpoint, irrespective of whether it is a physicochemical property, environmental fate parameter, human health effect, or ecotoxicological effect. In practice, however, it is not encouraged for basic physicochemical properties e.g. water solubility and log K_{ow}, since they provide key information for the assessment of a chemical. Reliable physicochemical data should normally be available or easily obtainable, and does not involve conducting *in vivo* animal studies.

Interpolation, extrapolation and uncertainty

As indicated above, the use of read-across as part of a category or analogue approach involves taking data from one or more substances in the group and applying it to the data-poor substance(s) within a category.

The OECD guidance and the ECHA guidance (OECD, 2007a; ECHA REACH TGD) on the use of read-across state that this can be done in one of two ways: interpolation or extrapolation.

Although these methods are described within the guidance on the use of read-across, they are briefly described again here.

> Interpolation: Within a category where trends in toxicity or factors influencing toxicity have been identified and the category members arranged in line with the trend (for example in order of increasing molecular weight), data from category members on either side of a data-poor category member can be used to predict its hazards.
>
> Extrapolation: Within a category where trends in toxicity or factors influencing toxicity have been identified and the category members arranged in line with the trend (for example in order of increasing molecular weight), data from category members at one end of the category can be used to predict the hazards of those members at the other end.

Within the guidance on the use of categories (OECD and REACH) there is a clear preference for the use of interpolation when performing read-across. Within the legal text for the EU REACH regulation (Annex XI, 1.5) (EU, 2007) the preference for interpolation goes further, with the text stating that read across from one substance to another as part of a category can be done via interpolation; there is no mention of extrapolation (although as indicated, the guidance does cover the use of both interpolation and extrapolation). It should be recognised that the predictions of many SAR models are actually extrapolations based on trends identified with structurally similar substances. Therefore, if the use of extrapolation is considered to be 'unacceptable' then so must the predictions from these models.

The preference for the use of interpolation comes from the assumption that it gives rise to less uncertainty than extrapolation. Extrapolation is therefore considered as less reliable due to this higher level of uncertainty associated with predictions. The exceptions to this (at least from a regulatory perspective) are those cases where extrapolation from one substance to another leads to an equally severe or more severe hazard assessment for the data-poor substance. For example, using extrapolation to read across skin sensitising data from one substance to another would be acceptable when the result is that both are classified as sensitisers.

Although it appears logical to state that interpolation is more 'acceptable' than extrapolation due to the perceived differences between the uncertainty associated with each method, one must be realistic in recognising that the degree of uncertainty is not due to the interpolation or extrapolation of data, but rather the robustness of the 'trend(s)' forming the basis of the category. This in turn is dependent on the size of the category and the amount of data on the members. If a trend is poorly defined or misunderstood then the use of interpolation is just as likely to give rise to significant uncertainty as extrapolation. For example, using the negative reproductive toxicity data on n-pentane and n-heptane to read across via interpolation to n-hexane would lead to an inaccurate assessment of its reproductive toxicity. On the other hand, extrapolating from the positive data on n-hexane to assess the reproductive toxicity of n-heptane would be inaccurate, but would be considered more acceptable as both substances would be classified as reproductive toxicants. Therefore, before excluding a particular methodology for reading across, one must first be confident that

trends have been accurately identified, characterised and defined. In large, data-rich categories there is a greater probability that the trends in toxicity can be identified and understood, therefore read-across using interpolation and extrapolation is more likely to be robust and acceptable. However, in cases where an analogue approach has been used or where a category consists of only a small number of members, trends can be far more difficult to identify and interpolation is often not possible simply due to the small number of members. In these situations extrapolation of data from one substance to another may be the only possibility for making use of read-across. In order to minimise uncertainty, a WoE can be built that incorporates the use of extrapolation in these cases. For example, using QSAR tools, *in vitro* assays or mechanistic/bridging studies to support the predictions from using read-across.

When utilising read-across, whether using interpolation or extrapolation, there is always some degree of uncertainty associated with predictions of hazards and toxicity. Indeed, it is perhaps worth noting that there are inherent uncertainties with all test data not just when applying read-across. One of the challenges is how to address this uncertainty during hazard characterisation. It can be considered that there are three types of toxicological endpoint. The first type includes endpoints where there is a 'presence/absence' of effect, for example sensitising potential, ready biodegradability, genotoxicity. The second type includes studies where a 'no effect level' is the result, for example the acute and repeated-dose toxicity studies (ecological and mammalian). The third type includes studies where the presence or absence of an effect is assessed along with the 'potency', for example reproductive toxicity, developmental toxicity, carcinogenicity etc.

The uncertainty associated with using read-across should be dealt with differently for each of these types of endpoints.

For the first type of endpoints, the standard approach should be to characterise the hazards of the data-poor substance in the same way as the substance providing the data. If the substance with data is not a sensitiser then the substance being read across would not be considered a sensitiser. The only time this approach should be deviated from is where there is a robust reason for characterising them differently.

For the second type of endpoints where a no effect level is the result, this level is often utilised in a risk assessment. This allows an opportunity to address uncertainty by the application of additional assessment factors when appropriate. If this approach is taken, it should be acknowledged that not all read-across situations introduce significant uncertainty. Therefore the default factor should be 1. For example, where there is a clear trend and sufficient data within the category on several members supporting the trend, and there is high confidence in the predicted no effect level, a factor of 1 can be used. However, where the trend is less defined or the quality of the data on the other members is sub-optimal, a higher factor could be used.

It then follows that for those endpoints where there is a combination of the presence/absence of an effect and potency (no effect level), i.e. the third study type, a combination of the above approaches could be taken.

With respect to classification, it is inappropriate to use a more conservative classification to address uncertainty in read-across. The classification and labelling systems such as the Globally Harmonised System should be based on an accurate assessment of the data with respect to the criteria. The classification information is intended to alert users to potential hazards associated with the handling and use of a

substance. Over-classifying a substance as a result of the use of read-across provides users with false information and should be avoided.

2.1.2 Trend analysis and computational methods based on internal models

For some category endpoints, the members may be related by a trend (e.g. increasing or decreasing molecular mass, carbon chain length or some other physicochemical property). When analysing trends in data, the following may be considered:

- The quality of the available experimental data based on the Klimisch rating system (Klimisch et al, 1997). For (Q)SAR and *in vitro* testing a similar scoring system is being developed (see Hulzebos and Gerner, 2010 for an example).
- Are there any inter-laboratory and inter-experiment variations (species, strains, endpoints, test protocols), and how do they affect data interpretation?
- The larger a category, the higher the likelihood that there may be breaks in trends which may affect the reliability of interpolation or extrapolation. In this case, it might be better to form subcategories with closely related trends**.**
- In instances where the members of a category do not form a simple homologous series, i.e where log K_{ow} could be used as a parameter to evaluate trends across the group, an appropriate alternative parameter should be established, e.g. a rate constant which characterises reactivity.
- Where multiple experimental data are available for a single substance, the result from an external (Q)SAR/expert system may assist in choosing a valid data point.
- Statistical power e.g. correlation, significance, etc.

A demonstration of a consistent trend in behaviour is often desirable as it may indicate a common mode of action. When some category members have measured values, and a consistent trend is observed, missing values may be estimated simply by scaling from the measured values to fill in the data gaps.

With a sufficiently large number of compounds in a category, and where a trend is observed, the data can be used to derive an internal (Q)SAR model that describes the properties of the members of the category. This has been most readily exemplified by trends in ecotoxicological properties such as aquatic acute toxicity in fish where the endpoint result has been a continuous variable e.g. LC_{50}. A (Q)SAR is a statistically established correlation relating (a) quantitative parameter(s) derived from chemical structure or determined by experimental chemistry to a quantitative measure of biological activity. (Q)SAR often take the form of regression equations, and can make predictions of effects/activities that are either on a continuous scale or on a categorical scale. This is one of the main functionalities available within the OECD (Q)SAR Toolbox.

A trend might also be expressed as a quantitative activity-activity relationship ((Q)AAR). A (Q)AAR is a mathematical relationship between two biological endpoints, which can be in the same or different species. (Q)AAR are based on the assumption that knowledge about the mechanism or mode of action, obtained for one endpoint, is applicable to the same endpoint in a different species, or to a similar endpoint in the same species, since the main underlying processes are the same (e.g. partitioning, reactivity, enzyme inhibition).

Thus, a chemical category can be seen as a set of internal (Q)SAR (and possibly also internal (Q)AAR) for the different endpoints, with the advantage that all the underlying data are transparently available to the assessor. Such models provide quantitative descriptions of the trends within a category and are referred to as internal (Q)SAR (or (Q)AAR) because they are derived directly from the experimental data for the category members. These models are also likely to be local models in the sense that they are based on relatively small datasets.

2.1.3 External (Q)SAR models and expert systems

For some endpoints, external (Q)SAR models or expert systems exist, and these can be used to fill the data gaps. Here the term external model is used in distinction to the internal model described in the section above and can refer to any model ((Q)SAR, QAAR or expert system) that was not developed as part of the category formation process. The data gap filling should only be done for compounds that fit into the applicability domain of the selected (Q)SAR model/expert system, i.e. an assessment with respect to the OECD validation principles of both the model and the prediction itself should be undertaken to determine robustness and relevance.

As defined by Dearden et al (1997) *"An expert system is any formal system, not necessarily computer-based, which enables a user to obtain rational predictions about the toxicity of chemicals. All expert systems for the prediction of toxicity are built upon experimental data representing one or more toxic manifestations of chemicals in biological systems (the database), and/or rules derived from such data (the rulebase)."* Examples of (Q)SAR tools and expert systems as encoded into software applications are discussed in detail in Chapter 3.

3. RESOURCES FOR NON-TESTING APPROACHES

There is a myriad of information in the public domain regarding non-testing approaches. Many resources exist including review papers on (Q)SAR and grouping, regulatory technical guidance documents, industry guidance documents, user fora e.g. OECD (Q)SAR Toolbox User Discussion Forum (https://community.oecd.org/community/toolbox_forum) as well as user guides for different software tools. An exhaustive compilation of all the resources is not provided within this report. Instead, a cross-section is described to provide a representative overview of both regulatory and non-regulatory guidance. Resources specific to particular endpoints are discussed in subsequent chapters as far as possible.

A number of sources of regulatory guidance are available to assist with approaches in the categorisation and grouping of chemicals. Certain of these are referenced and briefly reviewed below. This is not a comprehensive list but key common approaches and requirements are addressed by these guidance documents. REACH strongly advocates the use of alternative approaches including the use of *in vitro* methods, (Q)SAR and chemical categories as a means to satisfy the information requirement for risk assessment. Whilst technical guidance is available, there still remains little practical guidance about how alternative approaches can or should be applied in either the evaluation of existing (Q)SAR or in the formation of robust categories (Patlewicz et al, 2011).

3.1 Regulatory guidance

3.1.1 ECHA guidance

Currently the principle driver for the use of chemical class grouping and categorisation has been the implementation of REACH and the promotion of non-testing approaches. Paragraph 1 of Article 13 of the REACH regulation specifically permits the use of non-testing approaches when appropriate (EC, 2006). It is also specified in Annex XI that the generation of a comprehensive test dataset for every chemical is not needed if these test data can be replaced by practical non-testing and testing approaches, as a stand-alone method or in combination: *in vitro* methods, computational methods, optimised *in vivo* tests, chemical categories, read-across and exposure-based waiving/adaptation (EBW/EBA) (Van Leeuwen et al, 2007). In particular for human toxicity, information shall be generated whenever possible by means other than vertebrate animal tests, through the use of alternative methods, for example, *in vitro* methods or (Q)SAR models or from information from structurally-related substances (read-across). ECHA has developed a number of practical guides and guidance documents on the implementation of various aspects of the REACH regulation as described below.

In addition, a RAAF is currently being developed by ECHA for their internal use as a framework for evaluation. This will allow a structured and systematic review of registrant read-across supported submissions. The RAAF will address read-across at two tiers. Tier I is proposed to consist of a simple screening review for the rejection or further review of a read-across. If a read-across passes Tier I, then it may be reviewed at Tier II, where greater scientific rigour is applied and expert judgement will also be taken

from the Read-Across Expert Group (RAEG). The RAAF is still under development but is expected to be completed by the end of 2012.

Practical guide 6: How to report read-across and categories (ECHA, 2009)

This document presents a detailed overview on how to perform and report read-across and categories with particular focus on reporting within IUCLID. It provides practical guidance on how to develop a category and the basis for grouping chemicals of interest in terms of their similarity. It also recommends following guidance on identification and naming of substances under REACH for all category members, including UVCB substances. The Practical Guide also references the supplemental information requirements as discussed in the ECHA Chapter R.6 Guidance: (Q)SAR and the Grouping of Chemicals, described below.

Guidance on information requirements and chemical safety assessment. Chapter R.6: QSAR and grouping of chemicals (ECHA REACH TGD)

This document provides comprehensive details on the various approaches that may be adopted using (Q)SAR and grouping strategies. In the first part of the guidance, (Q)SAR and their application(s) are discussed, while in the second part the grouping of chemicals is addressed. This document describes two approaches for grouping to facilitate read-across: the 'analogue-approach', based on one source chemical, and the 'category approach', based on multiple source chemicals. The category approach allows the identification of trends across a category, improving the robustness of the read-across in cases where consistent data are available.

(Q)SAR guidance

Within the REACH framework the use of non-testing information may be used, if as required by Annex XI certain conditions can be satisfied:

- results are derived from a (Q)SAR model whose scientific validity has been established,
- the substance falls within the applicability domain of the (Q)SAR model,
- results are adequate for the purpose of classification and labelling and/or risk assessment and,
- adequate and reliable documentation of the applied method is provided.

The guidance discusses the use of (Q)SAR with reference to the OECD principles for (Q)SAR validation (see OECD 2007b below), a guiding principle is that a (Q)SAR should only be applied when the underlying OECD Principles for (Q)SAR validation can be met. Further discussion addresses the validity, reliability, applicability domains, adequacy and regulatory relevance of the (Q)SAR approach. Reporting formats for the models themselves ((Q)SAR model reporting format - QMRF) and for the model output ((Q)SAR prediction reporting format – QPRF) are provided.

The computational tools available ((Q)SAR and expert systems) are discussed. However, although this serves as an excellent introduction and resource due to the rapid development of this area, more recent reviews on their specific applicability may be more relevant. Tools for specific endpoints are referenced in the ECHA endpoint-specific guidance (ECHA REACH TGD). As mentioned already, a number of the programs available are discussed later in this chapter.

Chemical grouping

The second part of the guidance addresses grouping of chemicals. This is further subdivided to describe the two approaches for the grouping of chemicals:

1. The use of chemical categories with a large number of chemicals of similar physicochemical, human health and/or environmental toxicological and/or environmental fate properties. It is to be anticipated these similar properties are most likely to be founded on structural similarities.
2. The use of the analogue approach, where the number of chemicals in a group is much smaller or where trends are not obvious. For both approaches read-across may be used to fill data gaps and guidance is also offered on this.

Detailed discussion and guidance is provided on the benefits of the chemical category approach, underlying concepts including the mechanistic basis of chemical categories their application, robustness and relationship to (Q)SAR approaches. Less discussion is presented on the analogue approach. However for both approaches, analogue and category, stepwise guidance is provided on how these should be performed, including descriptive flow schemes and example reporting formats.

The reporting formats are presented and discussed with examples. To illustrate the use of the chemical category approach a case study using phosphonic acid compounds and alkali metal salts is presented.

3.1.2 OECD guidance

The OECD has produced a series of guidance documents on testing and assessment. These are comprehensive and also subject to periodic revision; a number of these are discussed.

OECD Series on Testing and Assessment No. 49 (OECD, 2004)

Report from the expert group on (quantitative) structure-activity relationships [(Q)SAR] on the principles for the validation of (Q)SAR.

In this report, the so-called Setubal Principles for (Q)SAR are discussed and developed into the OECD Principles for (Q)SAR validation for application in the regulatory use of (Q)SAR models.

To facilitate the consideration of a (Q)SAR model for regulatory purposes, it should be associated with the following information:

a) A defined endpoint;
b) an unambiguous algorithm;
c) a defined domain of applicability;
d) appropriate measures of goodness-of-fit, robustness and predictivity;
e) a mechanistic interpretation, if possible.

A checklist was developed to provide guidance on the interpretation of the OECD principles. These principles were then applied to a number of (Q)SAR models covering diverse toxicological and ecotoxicological endpoints. Note whilst the ECHA guidance makes reference to the OECD principles, it does stipulate additional conditions as far as use which are outlined in Annex XI.

OECD Series on Testing and Assessment No. 58 (OECD, 2006)

Report On The Regulatory Uses And Applications In OECD Member Countries Of (Quantitative) Structure-Activity Relationship [(Q)SAR] Models In The Assessment Of New And Existing Chemicals.

This review summarised the prevailing situation within OECD member countries and the regulatory use of (Q)SAR applications. The regulatory position at that time was presented via case studies for: Canada, Czech Republic, Denmark, Netherlands, USA (US Environmental Protection Agency (EPA), US Food and Drug Administration (FDA)) and the European Commission. In addition the PROSPECTIVE USES OF (Q)SAR WITHIN OECD MEMBER COUNTRIES was presented in a similar manner using case studies from: Australia, Germany (Federal Institute for Risk Assessment (BfR), Federal Environment Agency (UBA)), Italy, Japan, UK and the European Commission.

As mentioned in the foreword to the OECD review, it was anticipated that the document would be prospective in nature since it discussed not only the current regulatory use of (Q)SAR but also prospective uses due to the implementation of new chemical regulations e.g. REACH.

OECD Series on Testing and Assessment No. 69 (OECD, 2007b)

Guidance Document On The Validation Of (Quantitative) Structure-Activity Relationship [(Q)SAR] Models.

This provides comprehensive guidance on the validation of (Q)SAR models. Detailed discussion is presented across chapters covering the specific requirements for the above mentioned five OECD principles for validation.

OECD Series on Testing and Assessment No. 80 (OECD, 2007a)

Guidance on Grouping of Chemicals.

This presents a comprehensive review of guidance on the grouping of chemicals. However the guidance is essentially the same as that provided in 'Guidance on information requirements and chemical safety assessment. Chapter R.6: QSARs and grouping of chemicals' (ECHA REACH TGD) discussed previously. The development of this guidance was borne out of the REACH technical guidance preparation just published sooner.

It should be noted that the OECD guidance on grouping is currently under revision. The revision takes note of the outputs developed in the OECD Series on Testing and Assessment No. 138: Report of the Workshop on Using Mechanistic Information in Forming Chemical Categories (OECD, 2011). In the draft updated guidance the utility of the AOP approach is discussed.

The updated revision also lists those chemicals discussed at a SIDS (Screening Information Data Set) Initial Assessment Meeting (SIAM) and assigned to a chemical category.

OECD Series on Testing and Assessment No. 102 (OECD, 2009a)

Guidance document for using the OECD (Q)SAR Application Toolbox to Develop Chemical Categories according to the OECD Guidance on Grouping of Chemicals.

OECD Series on Testing and Assessment No. 101 (OECD, 2009b)

Report of the Workshop on Structural Alerts for the OECD (Q)SAR Application Toolbox.

OECD Series on Testing and Assessment No. 138 (OECD, 2011)

Report of the Workshop on Using Mechanistic Information in Forming Chemical Categories.

This report reviews the discussions and examples presented at a workshop on 'Using Mechanistic Information in Forming Chemical Categories', (2010, Crystal City VA, USA). The workshop discussed ways in which the OECD (Q)SAR Project and the Toolbox could benefit from the concept of the AOP. As defined in the report, *"an AOP delineates the documented, plausible, and testable processes by which a chemical induces molecular perturbations and the associated biological responses which describe how the molecular perturbations cause effects at the subcellular, cellular, tissue, organ, whole animal and (when required) population levels of observation"*.

The aims of the workshop were to:

- review the extent of the knowledge on mechanism or mode of action in the context of key events or processes that lead to specific adverse outcomes that are used in risk assessment;
- propose how scientific information on mechanism or mode of action can be organised as key events and processes within adverse outcome pathways to aid the formation of categories;
- examine a series of case studies using adverse outcome pathways;
- gather input on work flow(s) for using adverse outcome pathways to form chemical categories, and to gather input on the role of (Q)SAR methods in forming categories based on key events in an adverse outcome pathway.

The report presents a definition of the AOP concept. The concept is further described through the discussion of specific examples defining the key events at each step along the pathway. While the AOP approach may be more holistic in concept, the mode of action will be incorporated into the AOP.

The concepts of the AOP have been incorporated into the review of the OECD Guidance on Grouping (Revision of OECD Series on Testing and Assessment No. 80: Guidance on Grouping of Chemicals).

OECD Cooperative Chemicals Assessment Programme

In addition to the specific guidance documents on testing and assessment a broad programme, the OECD Cooperative Chemicals Assessment Programme, was initiated with the aim of covering a greater range of chemicals and assessments than simply just HPV chemicals. Central to this is the OECD Manual for the Assessment of Chemicals. The Manual has been updated with new versions of Chapters 1, 2, 4, 5 and 6, and is available from the OECD website (http://www.oecd.org /env/chemicalsafetyandbiosafety/assessmentofchemicals/manualfortheassessmentofchemicals.htm). The chapter contents are shown below. Chapter 3, on the guidance for grouping of chemicals is currently in draft revision. This should be read in conjunction with the guidance contained in the OECD Series on Testing and Assessment No. 80: Guidance on Grouping of Chemicals.

- Chapter 1 provides an introduction to the OECD Cooperative Chemicals Assessment Programme.
- Chapter 2 describes the process for the gathering of data.
- Chapter 3 contains guidance for evaluating the quality of data (Chapter 3.1) as well as guidance for grouping of substances (Chapter 3.2) and the use of structure-activity relationships (Chapter 3.3).
- Chapter 4 contains guidance for assessing the hazards of chemical substances to man and the environment.
- Chapter 5 describes how to elaborate the assessment report (also includes a template assessment report and explanatory notes).
- Chapter 6 describes how to elaborate the assessment profile (also includes a template assessment profile).

3.1.3 USA EPA High Production Volume (HPV) Challenge Program

The USA EPA High Production Volume (HPV) Challenge Program addresses chemicals *"produced or imported in the United States in quantities of 1 million pounds or more per year"* (http://www.epa.gov/hpv/).

The information that needs to be assembled under the Program including physicochemical, toxicological, environmental fate and ecotoxicology information is catalogued into the High Production Volume Information System (HPVIS). It allows the user a search using the Category Matrix Search facility. The category grouping allows the user to 'read-across' a category. The technical documentation refers to this and thus presents results for HPV Challenge Program data that were reported in categories of chemicals. The report is a matrix of the individual chemical members of the category as one axis and the HPVIS endpoints as the other. The intent of the report is to assist users in performing a 'read-across' analysis to estimate values for chemicals in the category without a result reported for a specific endpoint.

From the HPVIS, a number of hazard characterisations have been derived, based on the chemical grouping methods. The HPVIS data are available via the US EPA website as part of the Agency's commitment to increased transparency.

In addition, the US EPA has since rolled out the Existing Chemicals Program. The underlying strategy for this programme is:

1. Risk assessment and risk reduction.
2. Data collection and screening.
3. Public access to chemical data and information.

The data from this and other data transparency initiatives may be accessed from the US EPA via the Chemical Data Access Tool (CDAT), a web-based tool which facilitates access to health and safety information submitted to EPA under the TSCA.

3.1.4 Canada Substance Groupings Initiative

As stated on the Substance Groupings Initiative website *"The Government of Canada plans to assess and manage, where appropriate, the potential health and ecological risks associated with nine groups of substances"* (http://www.chemicalsubstanceschimiques.gc.ca/group/index-eng.php). This programme runs under the Canadian Environmental Protection Act (CEPA, 1999), which details the underlying hazard identification and risk management measures for existing substances. The motivation for the Initiative was the realisation that while new chemicals were subject to regulation approximately 23,000 existing chemicals were not and hence these were to be sorted or placed into 'categories' which would ultimately determine those categories requiring further assessment.

The groupings that have been proposed to date are:

- Aromatic azo- and benzidine-based substances.
- Boron-containing substances.
- Certain internationally classified substances with potential for exposure to individuals in Canada.
- Certain organic flame retardants.
- Cobalt-containing substances.
- Methylenediphenyl diisocyanates and diamines (MDI/MDA).
- Phthalates.
- Selenium-containing substances.
- Substituted diphenylamines.

The website also contains links to additional resources and factsheets on categorisation and grouping.

3.1.5 Japan HPV Challenge Program

The Japanese HPV Challenge Program allows the use of category approaches after identification of chemicals of interest (http://www.env.go.jp/en/chemi/hpv.html). It is expected the approaches adopted for other HPV programmes may be used.

3.1.6 China New Chemical Substance Notification

On 15th October 2010, a REACH-style directive that regulates the environmental risk and hazard of China's new chemical substances, 'Measures on Environmental Management of New Chemical Substances', came into effect, under the Ministry of Environmental Protection (MEP). This regulation adopts several of the same principles and concepts of the European regulation, and is known as 'China REACH'. A guidance document has also been published to accompany the directive. Data generated through the following sources are accepted for the notification of new chemical substances in China: test reports, published authoritative literature, authoritative database, and other non-testing methods such as (Q)SAR, read-across and expert opinion. However, the guidance notes that test reports are to be given greater weighting than other data sources. Data generated by non-testing methods are usually not accepted at initial stage of review unless the testing could not be conducted scientifically (MEP, 2010a; MEP Annex I, 2010b).

3.1.7 TSCA New Chemicals Program (NCP) Chemical Categories

A large number of categories are described in the TSCA New Chemicals Program (NCP) Chemical Categories (Office of Pollution Prevention and Toxics, U.S. EPA, 2010). The purpose of the categorisation is to present chemicals *"for which sufficient assessment experience has been accumulated so that hazard concerns and testing recommendations vary little from chemical to chemical within the category"*. Currently, there are a total of 56 categories. EPA periodically updates the Chemical Categories Report. The most recent updated version is the August 2010 version. See http://www.epa.gov/oppt/newchems/pubs/chemcat.htm for further information.

3.2 Scientific literature

In addition to the regulatory guidance there are many literature publications detailing aspects of read-across and (Q)SAR approaches. References specific to particular endpoints are discussed in a later chapter. A handful of examples are highlighted here.

SAR determinations for a wide range of chemicals were compiled by Tennant and Ashby (1991) based on their response in bacterial gene mutation assays and correlations to outcome in long-term carcinogenicity assays. The underlying hypothesis was based on that put forward by Miller and Miller (1977) who found that it was possible to rationalise the activity of a large majority of animal carcinogens at the time on the basis of their electrophilic potential. This later led to the development of what are often referred to as the 'Ashby-Tennant Alerts'. These alerts have formed the basis for many later (Q)SAR and SAR programs. The authors developed a composite molecule containing their alerts as an aid to classification of a chemical, by eye, based on the structural moieties it contained.

A same hypothesis was used to facilitate a review by Enoch and Cronin (2010) of available alerts for genotoxicity and rationalise the mechanisms on the basis of organic chemistry principles previously established by Aptula and Roberts (2006). The alerts identified were subsequently incorporated into the OECD (Q)SAR Toolbox as so-named DNA Binding profilers. Effectively these profilers characterise the

molecular initiating events (MIEs) for genotoxicity which are the first step in the formation of AOPs. The development of *in silico* chemical categories requires the use of comprehensive chemical profilers, which are based on rules developed from human expert knowledge about a given MIE (Enoch et al, 2011).

A category approach has been tested for reproductive toxicity for phthalates with a particular alkyl side chain length (C4-C6) (Fabjan et al, 2006). Enoch et al (2009) have investigated the use of 2D similarity indices available within Toxmatch software to form categories on 290 substances for teratogenicity using data FDA's TERIS system.

A chemical category approach has also been applied to macrocyclic fragrance ingredients (e.g. ketones and lactones/lactides) for aquatic risk assessment (Salvito et al, 2011) based on the likely metabolic pathway for biodegradation and on expected ecotoxicological modes of action.

Wu et al (2010) developed a systematic framework for assessing the suitability of candidate analogues for read-across or SAR-based toxicology assessment. The process is multistep and makes use of information on structural, reactivity, metabolic and physicochemical similarity, including the Ashby alerts referred to above. The authors fully describe their process and the elements of the decision process aided by a flow chart of the analogue identification and evaluation process and a decision tree for categorising the suitability of the analogues. Example analogues are presented. The methodology was subsequently evaluated using a set of 14 case studies (Blackburn et al, 2011).

Fundamental to developing grouping or categorisation for the development of subsequent (Q)SAR is the ability to determine similarity of a number of chemicals. 'Toxmatch' was developed on a freely available open source basis (EU Joint Research Centre-JRC) to facilitate the grouping of chemicals into categories and read-across, using 'similarity indices'. The software is described in more detail in Chapter 3.3.3 (Gallegos Saliner et al, 2008a; Patlewicz et al, 2008).

Schaafsma et al (2009) called for a more intensive dialogue between stakeholders on the acceptance of non-standard approaches in order to meet the objectives of REACH. Non-testing tools reduce direct costs but it is important to realise that deviations from standard practices may increase the indirect costs to industry and the authorities because of discussions regarding acceptance. Rila et al (2006) discussed in the workability of the guidance documents for category and read-across. van Leeuwen et al (2009) referred to the growing use of the chemical categories approach by regulatory agencies. A chemical category is generally considered to be a group of chemicals whose properties are likely to be similar or follow a regular pattern as a result of mechanism, mode of toxic action or structural similarity. The problem according to van Leeuwen et al is that there was no generally accepted method for measuring similarity, nor could there be one since similarity was context dependent.

Many HPV chemicals have limited toxicological data and therefore may require additional costly information, such as the two-generation reproductive toxicity test, the developmental toxicity test and additional mutagenicity tests. These three endpoints represent approximately 70% of the total testing costs for REACH. They also represent 70% of the total number of laboratory animals needed for REACH implementation. There is a need for an integrated/intelligent testing strategy (ITS) that combines the use of computational and read-across approaches, albeit in a cautious and judicious way, with available tissue culture methods as a basis for

risk assessment, in conjunction with metabolism and bio-kinetic studies (MacGregor et al, 2001). The strategy should be applied by being driven by exposure information, and not by a tick-box approach. Combes and Balls (2005) identified key elements of such an ITS for assessing chemical risk under REACH legislation.

A considerable amount of work has been carried out by the Joint Research Centre's European Chemicals Bureau (ECB). Gallegos Saliner et al (2005) developed a computational method to facilitate the classification of chemicals into similarity-based chemical categories, which would be useful for building (Q)SAR models and for refining chemical category proposals. Some of the practical experiences from the major regulatory agencies as well as the guidance developed by the OECD are summarised by Patlewicz (2005). The conclusion is that regulatory use of read-across/chemical categories is still quite limited. There is a clear need for the development of practical guidance to promote greater uptake of these types of approaches. Worth et al (2007) presented a perspective of how computational approaches could be used for the development of chemical categories and in the application of read-across. It also contains a compilation of case studies that were developed by the ECB during 2006-2007. These case studies were performed to explore the possible applications of computational methods in the assessment of chemicals. Further details of case studies that were used to shape the REACH guidance on chemical categories and read-across are also reported (Worth and Patlewicz, 2007).

In Patlewicz et al (2011), a selection of case studies were structured to illustrate the way in which non-testing approaches could be applied under REACH with particular focus on (Q)SAR and category approaches. A follow-up study was performed in 2012 (Patlewicz and Lander, 2012).

The role of AOPs as a framework for the development of mechanistically meaningful chemical categories is discussed by Schultz (2010) and highlighted with an example for skin sensitisation by Schultz et al (2011).

3.3 Software resources

Non-testing approaches or methods were terms borne out of the REACH Technical Guidance. They address (Q)SAR, SAR, expert systems and chemical grouping approaches. Over the years and in particular with the advent of REACH, there has been a tremendous drive to develop software tools and approaches that could assist in the application of non-testing methods for regulatory purposes. Some of these tools are freely available; examples include those of the Cefic LRI Toolbox (http://www.cefic-lri.org/lri-toolbox) and OECD Toolbox, whilst others are commercial tools. The available software tools serve many applications including the ability:

a) To identify groups of similar chemicals and express this similarity in qualitative and/or quantitative terms, thereby supporting the formation of chemical categories and the application of read-across;
b) to provide mechanistic information, thereby supporting the interpretation of experimental data;
c) to fill data gaps, thereby replacing/reducing the need for (animal) testing;
d) to supplement available test data, thereby supporting a WoE assessment;
e) to identify chemicals of potential concern, in order to guide or prioritise testing.

For the purposes of this chapter, the tools described are categorised as those whose primary role is to make predictions of specific endpoints ((Q)SAR/Expert systems), those that predict metabolism and those that facilitate grouping approaches including read-across (Grouping tools).

3.3.1 (Q)SAR/Expert systems

Expert systems containing (Q)SAR are commonly applied to make predictions of specific endpoints in lieu of experimental testing or as supporting information as part of a WoE approach. Thus, their primary function really addresses points c) and d) from the list above. A secondary role is in providing the anchor or the context of similarity to justify why a given pair or set of substances are expected to behave similarly in terms of their biological activity. Clearly structural similarity is only one criterion that helps to substantiate similarity in activity, more importantly the features or factors that drive that toxicity can be conveniently encoded by the descriptors or inputs of a (Q)SAR model. This is certainly the case for those (Q)SAR where the features or descriptors can be interpreted and rationalised in terms of the likely plausible mode of action (MOA). Thus, (Q)SAR/Expert system can also provide useful mechanistic information to help substantiate a qualitative/quantitative read-across. Examples of expert systems that are customised for specific endpoints are discussed in Chapter 6.5. Here, some of the commonly available expert systems that cover a range of different endpoints are highlighted and grouped in terms of their availability.

Freely available tools: expert systems

CAESAR/VEGA

CAESAR was an EU-funded project (http://www.caesar-project.eu) that sought to develop a series of models that would be specifically applicable for REACH in that they would be characterised in accordance with the OECD Validation Principles for (Q)SAR (as described in Chapter 3.1.2). The project saw the derivation of five statistical models covering the following endpoints: mutagenicity (Ames), carcinogenicity, developmental toxicity, skin sensitisation, and the bioconcentration factor. These models were implemented into open-source software and made available for online use via the web, or in the case of Ames mutagenicity and developmental toxicity as standalone programs. After the CAESAR project was completed, a new platform called VEGA (Virtual models for property Evaluation of chemicals within a Global Architecture) was launched (http://www.vega-qsar.eu/index.php). The models that form the basis for VEGA have been taken from CAESAR or T.E.S.T. (see below), or have been developed later by the contributors to VEGA. VEGA can be interrogated on-line to make predictions of Ames mutagenicity, carcinogenicity, developmental toxicity, skin sensitisation, bioconcentration factor, 96hr LC_{50} in fathead minnow and log K_{ow}. Alternatively a standalone client called VEGANIC (VEGA Non-Interactive Client) can be downloaded and used off-line. The models may be interrogated to make predictions for the aforementioned endpoints or the inputs used in the application of these models may be used to support a quantitative/qualitative read-across. Functionality is also being developed to enable users to derive their own models based on categorical or continuous data in tools called SARpy and CORAL (correlation and logic models).

T.E.S.T

The Toxicity Estimation Software Tool is an open-source application developed by the US EPA's National Risk Management Research Laboratory. T.E.S.T. allows users to estimate a range of toxicological and physical properties. The toxicological endpoints that are currently in the software include 96-hrs fathead minnow LC_{50}, 48-hrs *Daphnia magna* LC_{50}, *Tetrahymena pyriformis* 50% IGC_{50}, oral rat LD_{50}, bioconcentration factor, developmental toxicity, and Ames mutagenicity. The physical property endpoints include boiling point, flash point, surface tension, viscosity, density, water solubility, and thermal conductivity. Further information on the models, their training sets are described in the accompanying user guide (http://www.epa.gov/nrmrl/std/qsar/testuserguide.pdf). The tool is freely downloadable from the EPA website at: http://www.epa.gov/nrmrl/std/qsar/qsar.html#TEST.

EPI Suite

EPI (Estimation Programs Interface) Suite estimates a range of physicochemical properties, environmental fate parameters and ecotoxicity. It has been developed by the US EPA in collaboration with Syracuse Research Corporation (SRC). EPI Suite is available from the US EPA website: http://www.epa.gov/oppt/exposure/pubs/episuite.htm. The current version of EPI Suite is v4.1. The models available are described in brief. AOPWIN v1.92 estimates the rate constant for the atmospheric, gas-phase reaction between photo-chemically produced hydroxyl radicals and organic chemicals. It also estimates the rate constant for the gas-phase reaction between ozone and olefinic/acetylenic compounds. The rate constants estimated by AOPWIN are used to calculate atmospheric half-lives for organic compounds based upon average atmospheric concentrations of hydroxyl radicals and ozone. KOWWIN v1.68 estimates the logarithmic octanol-water partition coefficient (log K_{ow}) of organic compounds based on 'fragment constant' methodology. BIOWIN estimates the probability of rapid aerobic and anaerobic biodegradation of an organic compound in the presence of mixed populations of environmental microorganisms. MPBPwin v1.43 comprises algorithms for the prediction of boiling point, melting point and vapour pressure. WSKOWWIN v1.42 and WATERNT v1.01 estimate the water solubility (WSol) of an organic compound using either a log K_{ow} or 'fragment constant' method. HENRYWIN v3.2 estimates the Henry's Law Constant of organic compounds over a temperature range (0^{o} to 50^{o} C) using one of two different algorithms known as the Bond Contribution Method and Group Contribution Method. KOAWIN v1.0 estimates the octanol-air partition coefficient (K_{OA}) of an organic compound using the compound's log K_{ow} and Henry's Law constant (HLC). KOCWIN v2.0 is a Soil Adsorption Coefficient Program which estimates the soil adsorption coefficient (K_{oc}) of organic compounds. BCFBAF v3.01 estimates the bioconcentration factor (BCF) of an organic compound log K_{ow}. In addition, algorithms for the prediction of the biotransformation rate (kM) in fish and the bioaccumulation factor (BAF) by the Arnot-Gobas method (Arnot and Gobas, 2003) are available. The inclusion of metabolism in the calculation of BCF is an important development in this version of BCFBAF. HYDROWIN v2.0 estimates aqueous hydrolysis rate constants for over 30 different chemical classes including examples such as esters, carbamates, epoxides, halomethanes, selected alkyl halides and phosphorus esters. HYDROWIN estimates acid- and base-catalysed rate constants which are then used to calculate hydrolysis half-lives at selected pH values. The other notable model that is incorporated in EPI Suite v4.1 is ECOSAR, an expert system of SARs for at least three aquatic species for over 120 different chemical classes. ECOSAR's current version v1.11 is available as a standalone tool. ECOSAR v1.0 is still integrated in EPI Suite v4.1. The standard profile typically contains three acute values, and three chronic values for fish, daphnid, and green

algae. Models rely on log K_{ow} for derivation of these acute or chronic values. The log K_{ow} values are estimated using KOWWIN v1.68.

SPARC

SPARC (SPARC Performs Automated Reasoning in Chemistry; supplied by ARChem) uses computational algorithms based on fundamental chemical structure theory to estimate a wide variety of reactivity parameters strictly from molecular structure. Parameters include pKa, vapour pressure, boiling point, diffusion coefficient (air and water) as a function of temperature and pressure, molecular volume as a function of temperature, polarisability, solubility, log K_{ow} amongst others. Further information can be found on the website at http://archemcalc.com/sparc.php.

Toxtree

Toxtree is a flexible and user-friendly open-source platform that encodes a number of rulebases for the evaluation of toxicity. It was originally commissioned by the JRC to encode the Cramer et al (1978) structural classes that are routinely used as part of a thresholds of toxicological concern (TTC) approach (Patlewicz et al, 2008). Since that time, Toxtree has been extended and further developed with other rulebases not only by its original developer, Ideaconsult Ltd (Sofia, Bulgaria) but by other consultants. It is freely available as a download from the JRC website:
http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/qsar_tools/toxtree

The current version Toxtree v 2.5 (August 2011) includes a broad range of schemes:

- Cramer scheme (Cramer et al, 1978) and an extended Cramer scheme.
- Mutagenicity and carcinogenicity rulebase known as the Benigni-Bossa rulebase (Benigni et al, 2008) as well as the ToxMic rulebase (Benigni et al, 2009) on the *in vivo* micronucleus assay.
- Skin sensitisation alerts based on the organic chemistry reaction principles published by Aptula and Roberts (2006) and encoded as SMARTS by Enoch et al (2008).
- SMARTCyp, a two dimensional method for the prediction of cytochrome P450-mediated metabolism (Rydberg et al, 2010), SMARTCyp predicts which sites in a molecule are labile for metabolism by Cytochromes P450.
- Verhaar scheme for the MOA of aquatic acute toxicity to fish (Verhaar et al, 1992; 1995; 2000).
- BfR and SICRET rules to predict skin irritation and corrosion (Walker et al, 2005).
- BfR rules to predict eye irritation and corrosion (Gerner et al, 2005).
- Kroes et al (2004) The International Life Sciences Institute (ILSI) decision tree for the application of the TTC approach.
- START biodegradability, a set of structural alerts compiled by the Canadian EPA for estimating the biodegradability potential of a chemical compound based on structural alerts.
- Michael acceptor profiler which identifies potential Michael acceptors based on structural alerts (Schultz et al, 2007).

Some of the rulebases encoded in Toxtree have since been implemented or re-encoded into the OECD (Q)SAR Toolbox.

In essence, Toxtree is effectively an expert system of SARs that can be useful to identify potential hazards but it also plays a useful role in providing the mechanistic information to substantiate read-across as part of a chemical group.

OpenTox

OpenTox (http://www.opentox.org) is an EU FP7 project on the development of Open Standards and an Open Source platform for predictive toxicology. The goal of OpenTox is to develop an interoperable predictive toxicology framework which may be used as an enabling platform for the creation of predictive toxicology applications. The overall project has many activities, but notable for this report are two applications that have been developed as prototypes, i.e. ToxPredict and ToxCreate. ToxPredict is an open-source web platform containing many different models. Some of the models are in common with those encoded in ToxTree v2.5 since one of the contributors and developers of OpenTox is Ideaconsult Ltd (Sofia, Bulgaria). Other tools include developers' own models for acute aquatic toxicity in fish, the OECD chemical categories, Caco-2 models for oral absorption, Lipinksi's rule of 5 as well as the toolbox of Lazar models (see below). ToxCreate, on the other hand, is an application for users to develop their own models using the suite of data mining tools and descriptor calculators made available on the website.

Lazar

Lazy Structure- Activity Relationships (Lazar) is an open-source software program that makes predictions of a range of endpoints using data published by DSSTox. Distributed Structure-Searchable Toxicity (DSSTox) Database Network (http://www.epa.gov/ncct/DSSTox/) is a project of EPA's National Center for Computational Toxicology, helping to build a public data foundation for improved structure-activity and predictive toxicology capabilities. The DSSTox website provides a public forum for publishing downloadable, structure-searchable, standardised chemical structure files associated with chemical inventories or toxicity data sets of environmental relevance. The Lazar models include the following endpoints: Ames mutagenicity, rodent and hamster carcinogenicity, Maximum Recommended Daily Dose (MRDD) and fathead minnow acute aquatic toxicity. The models are based on the use of statistical algorithms for classification (k-nearest neighbours and kernel models) and regression (multi-linear regression and kernel models). Lazar performs an estimation of applicability domain, provides a confidence index for each prediction and presents the nearest neighbours within the training set with their similarity indices and experimental data to provide additional context and confidence in the prediction.

A web-based prototype is accessible at http://lazar.in-silico.de. The models developed by Lazar are also available as part of the OpenTox project (http://www.opentox.org).

OncoLogic™

This is an expert system that assesses the potential of chemicals to cause cancer. OncoLogic™ was developed by the US EPA in collaboration with LogiChem, Inc. It estimates the likely level of concern for carcinogenicity by applying the rules of SAR analysis and incorporating what is known about the mechanisms of action and human epidemiological studies. The Cancer Expert System is composed of four subsystems that evaluate fibres, metals, polymers, and organic chemicals of diverse chemical structures. Chemicals are

entered individually and the user needs some knowledge of chemistry in order to select the appropriate structural class (of the 48 or so that are available) in order to make an assessment.

There are six concern levels for carcinogenicity within Oncologic™:

- Low - Unlikely to be carcinogenic.
- Marginal - Likely to have equivocal carcinogenic activity or may be weakly carcinogenic at doses at or exceeding maximum tolerated doses.
- Low-Moderate - Likely to be weakly carcinogenic, or carcinogenic toward a single target/species, or carcinogenic at relatively high doses.
- Moderate - Likely to be a moderately active carcinogen toward one or more target/species.
- High-Moderate - Highly likely to be a moderately active carcinogen toward one or more target/species.
- High - Highly likely to be a potent carcinogen even at relatively low doses, or carcinogenic toward multiple targets/species.

OncoLogic is freely downloadable from the US EPA website:
http://www.epa.gov/oppt/sf/pubs/oncologic.htm.

The structural classes have also been encoded as fully as possible into the OECD (Q)SAR Toolbox as one of endpoint profilers.

Commercially available tools

TOPKAT

TOPKAT (http://accelrys.com) is a commercial product of Accelrys Inc. that assesses the toxicity of chemicals from 2D molecular structure. (Q)SAR models (so called submodels) are available for different chemical classes and the program automatically selects the equation from the structural input. TOPKAT also makes visible experimental test data for similar analogues if available (taken from the respective training set). For each model, a model-specific similarity distance between a query structure and a database compound can be calculated. TOPKAT is able to make predictions for a wide range of endpoints. It is available as a module within Pipeline Pilot and Discovery Studio as part of the portfolio of Accelrys tools rather than a stand-alone program. Endpoints covered include aerobic biodegradability, log K_{ow}, acute aquatic toxicity to fathead minnow, acute aquatic toxicity to *D. magna*, FDA and NTP (National Toxicology Program) models for carcinogenicity in mice and rats (both sexes as well as single/multiple tumour sites), WoE carcinogenicity model, Ames mutagenicity, developmental toxicity, rat oral LD_{50}, maximum tolerated dose (MTD) in rats based on feed/water and gavage dosing regimens, chronic lowest observed adverse effect level (LOAEL), skin irritation (negative/mild vs. severe/moderate), eye irritation (to discriminate between severe/moderate and mild/non-irritating as well as to discriminate between severe/moderate or mild/non-irritating), skin sensitisation (presence/absence of sensitising potential as well as potency (severe vs. mild/moderate sensitisers). At one time TOPKAT was only available as a standalone program, currently with its integration as a component in the Discovery Studio suite (also can be utilised within Pipeline Pilot) affords greater flexibility

of the models themselves. Rather than the tools being fixed for predictions, users can now integrate their own data and information and refine and extend the scope of the modelled endpoints.

Derek Nexus

DEREK (Deductive Estimation of Risk from Existing Knowledge) predicts a number of human toxicological endpoints (https://www.lhasalimited.org/index.php/derek_nexus/). Derek Nexus contains 64 new Rapid Prototype alerts which are structural fragments for multiple endpoints and identify adverse effects and alerts the user to their presence. Derek Nexus presents the qualitative likelihood of an effect to occur. An effect may be certain, probable, plausible, equivocal, doubted, improbable, impossible, open, contradicted. In case these qualitative predictions are presented, it can be assumed that the query substance is in its applicability domain. The program may also give rise to predictions denoted as 'Nothing to report'. In these cases, the substance being in or outside the applicability domain is more difficult to evaluate (e.g. Hulzebos and Gerner, 2010). Derek Nexus' main focus lies in the areas of skin sensitisation, mutagenicity, carcinogenicity. Structural fragments and rules are also available for irritation endpoints, organ toxicity, photosensitisation and methaemoglobinia. For skin sensitisation, skin permeability parameters are included to refine the prediction. In general, effects are predicted for both humans and mammals if differences can be distinguished. Derek Nexus provides arguments on the MOA along with the predictions. Examples and references on which predictions are based are supplied as and when available (e.g. Hulzebos and Posthumus, 2003; Fuart-Gatnik and Worth, 2010).

MultiCASE

MultiCASE Inc. (http://multicase.com/), implements the so-called CASE (Computer Automated Structure Evaluation) approach, and is referred to in different ways (MCASE or MC4PC), depending on the software version and computer platform and its successor. The program automatically generates predictive models from datasets provided by the user. It is based on a fragment-based technology sometimes referred to as the CASE approach (Klopman and Rosenkranz, 1995). The program performs a hierarchical statistical analysis of a database to discover substructures that appear mostly in active molecules thus being with high probability responsible for the observed activity. Initially, it identifies the statistically most significant substructure within the training set. This fragment, labelled the top biophore, is considered responsible for the activity of the largest possible number of active molecules. The active molecules containing this biophore are then removed from the database, and the remaining ones are submitted to a new analysis for identification of the next biophore. The procedure is repeated until either the activity of all the molecules in the training set has been accounted for or no additional statistically significant substructure can be found. Then for each set of molecules containing a specific biophore, the program identifies additional parameters called modulators, which can be used to derive (Q)SAR within the reduced set of congeneric molecules. The modulators consist of certain substructures or physicochemical parameters that significantly enhance or diminish the activity attributable to the biophore. (Q)SAR are then derived by incorporating the biophores and the modulators into the model. The program includes modules to predict physicochemical properties and a range of toxicological endpoints, including carcinogenicity, mutagenicity, teratogenicity, irritation, developmental toxicity, and acute toxicity. For the endpoints, the software uses its own toxicity scale, from 0 to 100 CASE units, to cover the range from inactive, marginally active and active. In many cases, it is difficult to relate

these CASE units to traditional measures of toxicity. Predictions generated from MCASE models have been collected by the Danish EPA and are available within the OECD (Q)SAR Toolbox.

TIMES

The Tissue MEtabolism Simulator (TIMES), developed by LMC (Bourgas University, Bulgaria; http://oasis-lmc.org/) is a platform which encodes structure toxicity and structure metabolism relationships through a number of transformations simulating metabolism and interaction of the generated reactive metabolites with cellular nucleophiles. The metabolism simulators mimic metabolism using 2D structural information. Metabolic pathways are generated based on a set of hierarchically ordered principal transformations including spontaneous reactions and enzyme-catalysed reactions (phase I and II). The covalent reactions with nucleophiles are described by alerting groups (structural alerts). Some of these alerts are additionally underpinned by mechanistically based 3D-QSARs to refine the predictions. These 3D-QSAR models depend on both the structural alert and factors that influence its reactivity – steric effects, molecular size, shape, solubility, lipophilicity and electronic properties. The models within TIMES that are driven by metabolism simulators are those for Ames mutagenicity, *in vitro* chromosomal aberration, skin sensitisation and oestrogen-receptor affinity. Work is on-going to develop and refine a model for the *in vivo* micronucleus assay. Other models that are incorporated within TIMES but which do not necessarily account for metabolic transformation include models for receptor-binding affinities (oestrogen, androgen and aryl hydrocarbon receptors), phototoxicity as well as acute aquatic toxicity to different species including fathead minnow, *daphnia* and microorganisms such as *T.-pyriformis*.

Catalogic

A sister platform to TIMES, also developed by LMC covers environmental fate properties notably biodegradation according to different OECD Test guidelines (301C, 301B, 301F in OECD, 1992). Models also exist for bioconcentration and half-life in fish. Catalogic models incorporate a microbial metabolism simulator.

The metabolism simulators encoded within the TIMES and Catalogic tools are additionally available within the OECD (Q)SAR Toolbox. Metabolic trees, quantities, probability or schemes are not presented but the predicted metabolites formed are shown.

ACD/ToxSuite (ACD/Percepta)

The ACD/Tox Suite (formerly called ToxBoxes), provided by ACD/Labs (which merged with Pharma Algorithms in 2009), provides predictions of various toxicity endpoints including genotoxicity, acute toxicity, aquatic toxicity, eye/skin irritation and endocrine system disruption (http://www.acdlabs.com/products/pc_admet/tox/tox/). The predictions are associated with confidence intervals and probabilities, thereby providing a numerical expression of prediction reliability. The software incorporates the ability to identify and visualise specific structural toxicophores, giving insight as to which parts of the molecule are responsible for the toxic effect. It also identifies analogues from its training set, which can increase confidence in the prediction. The algorithms and datasets are not disclosed.

ACD/Tox Suite has been replaced by ACD/Percepta (http://acdlabs.com/products/percepta/), and ACD ToxSuite will be gradually phased out within the next two years (by end of 2014). ACD/Percepta unifies the software platforms for the physicochemical, ADME and toxicity prediction modules, and has better reporting abilities. It also offers the possibilities to view the calculation protocols, integrate easily with other software platforms, as well as unrestricted access to all information from individual prediction models, as well as from the consensus model (for physicochemical predictions).

3.3.2 Prediction of metabolism

Freely available tools

Metaprint2D

MetaPrint2D is a software tool (Bender et al, 2004) implementing a data-mining approach for predicting sites of xenobiotic metabolism. The algorithm is based on a statistical analysis of the occurrences of atom-centred circular fingerprints in both substrates and metabolites. This approach has undergone extensive evaluation and is able to make rapid predictions enabling users to explore the effects of structural modifications on a compound's metabolism in a responsive and interactive manner. MetaPrint2D is able to assign a confidence score to the predictions it generates, based on the availability of relevant data and the degree to which a compound is modelled by the algorithm.

A metric for assessing the performance of site of metabolism predictions was introduced to overcome any bias introduced by molecule size and the number of sites of metabolism.

This data-mining approach to site of metabolism prediction has been augmented by a set of reaction-type definitions to produce MetaPrint2D-React, enabling prediction of the types of transformations a compound is likely to undergo and the metabolites that are formed. This approach has been evaluated against both historical data and metabolic schemes reported in a number of recently published studies. Results suggest that the ability of this method to predict metabolic transformations is highly dependent on the relevance of the training set data to the query compounds.

MetaPrint2D has been released as an open source software library, and both MetaPrint2D and MetaPrint2D-React are available for use through the Unilever Centre for Molecular Science Informatics website (http://www-metaprint2d.ch.cam.ac.uk/).

CRAFT

Chemical Reactivity and Fate tool (CRAFT) is a software suite that has been designed to assist scientists in the area of product safety, hazard and risk assessment and toxicology to interactively evaluate the chemical reactivity, persistence, biodegradation and fate of chemical compounds in the environment. CRAFT provides decision support to faster estimate the environmental impact of existing chemicals or to discover and optimise better and safer new chemical entities and products.

Features and functionality:

- Generation and evaluation of biodegradation products of chemical substances in the environment.
- Generation of degradation trees and pathways weighted by the likelihood of occurrence of the individual reaction steps.
- Built-in knowledge base of chemical reactivity and biodegradation derived from the University of Minnesota Biocatalysis and Biodegradation Database.
- Extendible knowledge base to include user-defined models and rules for chemical reactivity and biodegradation.
- Flexible design to plug-in third party tools or to call external applications.
- Optional usage of on-line data sources to collect information about analysed chemicals, e.g. PubChem.
- Generation of printable and editable reports with user-definable level of detail.
- Open-source application available under LGPL licensing terms.

More detailed information is available from the developer's website: http://www.molecular-networks.com/products/craft.

Commercially available tools

Meteor

Meteor predicts the metabolic fate of xenobiotics using expert knowledge-based software through a comprehensive system of biotransformations and reasoning rules (https://www.lhasalimited.org/meteor/). The reasoning framework assigns a specific likelihood to the potential metabolites and has been carefully designed to allow users to prioritise biotransformation searches and extract the most likely metabolites from all the possible outcomes. The absolute reasoning rules are developed based on factors such occurrence ratios, species, log K_{ow} and molecular weight. Also taken into consideration is the depth within the metabolic sequence for which the query substrate occurs, i.e. whether the substrate is the administered compound, or a generated metabolite from another biotransformation.

To prioritise the most useful biotransformations, the confidence of a prediction can be assigned a specific likelihood based on the parameters mentioned above for Derek Nexus, starting at 'probable down through plausible, equivocal, doubted to improbable' (O'Leary-Steele and Long, 2011).

META

The META system is a commercially available tool developed by Klopman and co-workers (Klopman et al, 1994) at Case Western Reserve University (OH, USA). It is an expert system capable of predicting the sites of potential enzymatic attack and the nature of the chemicals formed by such metabolic transformations. The program uses dictionaries of biotransformation operators which are created by experts in the field of xenobiotic metabolism to represent known metabolic pathways. A query structure is entered and the program applies biotransformation operators according to the functional groups detected. After each biotransformation a stability check is performed on the reaction product by using quantum mechanical

calculations to detect unstable atom arrangements. The program then evaluates the stable metabolites formed and attempts to transform them further until water soluble metabolites that are deemed to be excretable are formed.

Accelrys Metabolite Database

Accelrys Metabolite Database (formerly known as MDL Metabolite, then Symyx Metabolite, then renamed to Accelrys Metabolite as a result of company merger between Symyx and Accelrys in 2010) is a comprehensive collection of xenobiotic transformations, compiled from the primary literature, conference proceedings, and NDAs; includes a browser for searching and displaying metabolic schemes and the functionality to archive one's own proprietary studies. More information is available under: http://accelrys.com/products/databases/bioactivity/metabolite.html.

METAPATH

METAPATH (http://oasis-lmc.org/?section=software&swid=3) developed by LMC (Bourgas, Bulgaria) is a searchable information management system for metabolism information including complete metabolic maps, experimental conditions, biotransformations, enzymatic system, rates and identified metabolites. It is currently being piloted within the OECD pesticides working group. The platform is accompanied with the biodegradation and liver metabolism databases which are being continually updated. Predictions of metabolism are implemented as metabolism simulators within LMC's other tools including TIMES and Catalogic and to a more limited extent within the OECD (Q)SAR Toolbox.

3.3.3 Grouping tools

There are a number of tools that have been developed that play a strong role in facilitating grouping approaches. The two most notable examples are Toxmatch and the OECD (Q)SAR Toolbox. These were both developed specifically to facilitate the development, evaluation, justification and documentation of chemical categories and read-across for regulatory purposes (ECHA REACH TGD).

Toxmatch

Toxmatch was developed by Ideaconsult under the terms of a JRC contract (Patlewicz et al, 2008). The current version is v1.07 and is available for free download from the JRC website http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/qsar_tools/toxmatch. The tool was developed as a chemical similarity tool, aimed at facilitating endpoint-specific quantitative read-across using similarity indices. The tool allows datasets to be compared either on the basis of structural features or on the basis of numerical descriptors. The comparison is executed using similarity indices. To promote the notion of relative similarity, i.e. similarity with respect to a given endpoint, Toxmatch made available several endpoint test sets. Included are datasets for skin sensitisation, skin irritation, acute aquatic toxicity to fathead minnow and bioaccumulation. Procedurally, a training set of substances is introduced into the system together with its endpoint result and relevant descriptors (either numeric or structural). A similarity index is chosen from a list of several that have been implemented into the program. This then creates by data mining techniques a

predictive model of the endpoint on the basis of the similarity index. A pairwise similarity matrix is also generated that profiles the diversity of the chemicals within the training set. A substance of interest or set of substances of interest is then introduced into the system as a test set. The similarity approach used for the training set is then applied to the test set. This generates a prediction of the endpoint for the substances of interest and enables the user to profile which substances are most similar on the basis of the similarity index used. The prediction is effectively a quantitative read-across and the exact algorithm differs depending on the nature of the endpoint result that was included in the original training set. For example, if the endpoint is categorical in nature, the similarity index identifies whether the nearest neighbours (default of 10) reside into one category or another. If the endpoint is continuous in nature, the estimate is a weighted average based on the nearest neighbours, where the similarity index weights the endpoint result and the sum of these weighted values give rise to the final prediction value. The default for the number of nearest neighbours is 10.

The tool is perhaps most effective at identifying structurally similar analogues from various training sets. For example, one notable use as described in Patlewicz et al (2011) shows Toxmatch being particularly effective at identifying analogues from the KOWWIN training set to substantiate a log K_{ow} prediction. This was made for a given substance that can be incorporated into the QPRF required for REACH for (Q)SAR predictions. The pairwise similarity index provided a convenient means of identifying structurally-related analogues from an available dataset.

OECD (Q)SAR Toolbox

The OECD (Q)SAR Toolbox was first launched as a pilot in 2008. It was developed to mimic the category workflow that is described in the current Chapter R.6 of the REACH technical guidance (ECHA REACH TGD) and the current OECD grouping document (OECD, 2007a). The intention was that this tool would facilitate the practical development, evaluation, justification and documentation of chemical categories and read-across. The most recent version now available is Toolbox v2.3. This is available for free download from the OECD website (see http://www.oecd.org/document/54/0,3746,en_2649_34379_42923638_1_1_1_1,00.html#Download_qsar_application_toolbox).

This is the penultimate version of the tool now in its Phase 2 of development, a 4-year project that was funded by ECHA. Version 3.0 will be released in October 2012. The OECD (Q)SAR Toolbox has a wealth of functionality. It contains various regulatory inventories as well as donations of experimental data. Its extensive profiling library enables a search of relevant analogues to be performed on the basis of similarity indices or on the basis of chemical functionality or on the basis of SAR information relevant for a given activity. This allows pragmatic groups to be formed and refined depending on available data and relevant MOA similarity. Profiling capabilities include SAR rulebases for skin sensitisation, mutagenicity, aquatic acute toxicity, bioaccumulation, biodegradation and other. Profilers also exist to simulate likely metabolites formed through biodegradation processes or as a result of skin/liver metabolism. Depending on available experimental data, data gaps can then be filled using read-across (a qualitative read-across is performed), trend analysis (a fitted line plot is derived) or by external (Q)SAR (several external (Q)SAR such as the Danish EPA's (Q)SAR or the EPIWIN suite of tools are implemented into the Toolbox). Once a prediction is made to fill a data gap, the Toolbox is able to save the model generated and report the prediction in the standardised templates described in both the REACH and OECD guidance. The most recent version of the Toolbox has

extensive import/export functionalities that allow integration with IUCLID 5.3. The data that is contained within the Toolbox is structured in accordance with the OECD global harmonised templates that are the cornerstone of IUCLID 5. This means that analysis can be conducted within the Toolbox and exported directly into IUCLID to minimise the manual manipulation of the data entry within IUCLID since the relevant fields are prepopulated. For this reason, the Toolbox is being heavily promoted for use under REACH. The current developers of the Toolbox have also worked to extend the capabilities of the Toolbox to integrate with other third party tools. Their own tools namely TIMES, Catalogic discussed already are now included as part of the set of external (Q)SAR models that can be invoked to generate predictions and for the outputs to be formatted into the QPRF documents required and appended within IUCLID.

Current work on the OECD (Q)SAR Toolbox is being focused towards handling of tautomers, metabolites, and mixtures.

The OECD (Q)SAR Toolbox thus is a useful tool to fill data gaps through the development of endpoint-specific groupings. It is also useful as a source of SAR rulebases that can be used to profile substances on the basis of their similar attributes and evaluate the context of similarity with respect to specific endpoints and across endpoints. In this way, some of the capabilities of the Toolbox are not unlike knowledge-based expert systems that encode SARs such as Derek Nexus or Toxtree.

AMBIT

AMBIT, a software for cheminformatic data management, is an outcome of a CEFIC LRI-sponsored project and consists of a database and functional modules allowing a variety of flexible searches and mining of the data stored in the database (http://www.cefic-lri.org/lri-toolbox/ambit). The AMBIT database stores more than 450,000 chemical structures and their identifiers such as CAS (Chemical Abstracts Service), EINECS (European INventory of Existing Commercial chemical Substances), INChI (International Chemical Identifier) numbers. It also contains attributes such as molecular descriptors, experimental data together with test descriptions, literature references. The data, which is quality assured and organised in searchable templates, offers unique features on chemicals information (structure, data, text), including REACH applicable persistence, bioaccumulation, toxicity (PBT) and analogues assessment. AMBIT performs chemical grouping and assesses the applicability domain of a (Q)SAR offering a variety of methods including the use of different approached to similarity assessments. AMBIT is freely available (http://ambit.sourceforge.net/).

Leadscope

Other grouping tools available have not necessarily been developed with REACH in mind but are useful tools for data mining and SAR/(Q)SAR development. A particular example is the commercial tool Leadscope which is a data management and data mining tool developed and commercialised by Leadscope Inc (http://www.leadscope.com/). Leadscope in many respects pioneered the notion of controlled vocabularies and ontologies for toxicity endpoints and developed so-called ToxML as a means to structure and store toxicity data in a systematic manner for effective search and retrieval. The Leadscope tool can be licensed with access to databases that have been collated and structured using the ToxML format. Examples include the FDA databases for reproductive/developmental toxicity, genetic toxicity, carcinogenicity. Leadscope thus permits the search of related analogues on the basis of structural similarity but with available toxicity

information. This allows a bottom-up search of analogues to formulate chemical categories. Leadscope's other key functionality is its means of exploring and evaluating substances owing to the structural features that are implemented. Some 27,000 chemical fingerprints (medicinal chemistry structural features) are encoded into the tool. This is very effective for exploring existing datasets to extract potential SARs or for exploring and analysing one's own datasets to identify whether specific insights can be derived that can provide in-house SAR knowledge that would not necessarily be encoded into existing (commercially) available expert systems. The 27,000 fragments have another application, to enable the top down grouping of substances (discussed in more detail in Chapter 4). A large inventory or library of chemicals can be readily profiled on the basis of structural features and then clustered to formulate initial pragmatic groupings of substances that can be further evaluated. This is particularly useful in screening/prioritising/ranking exercises to identify strategic chemicals to test or to identify which chemicals merit further evaluation.

Pipeline Pilot

Scitegic's Pipeline Pilot is a sophisticated data mining application that comprises a set of tools to analyse and visualise data, build workflows to manipulate structures and data, as well as a host of other (Q)SAR-related algorithms. Scitegic is a subsidiary of Accelrys Inc. The software's key feature is in automating data manipulation. Pipeline Pilot can retrieve or join data from independent databases, files, or other applications. It can be used directly to read chemistry, sequence, text, and numeric data from all popular formats and analyse data from multiple sources in real-time, without the need to first create a centralised database. Data can be easily manipulated through building up workflows from available components. These provide functionality for chemical processing, statistics, modelling, clustering, and reporting. The components allow structures to be modified, molecular properties and fingerprints to be calculated, (Q)SAR models to be derived, compounds to be clustered and maximal common substructures to be extracted. Thus, the capabilities within Pipeline lend itself to many of the same functionalities as Leadscope offers in terms of top-down grouping exercises.

DART

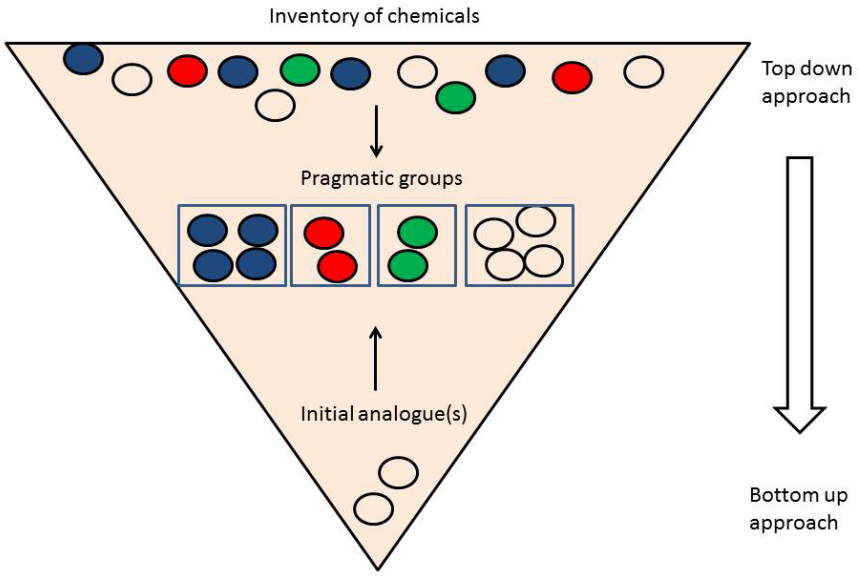
DART (Decision Analysis by Ranking Techniques), developed by Talete srl (Milan, Italy) under a JRC contract is a tool that can facilitate grouping of chemicals for ranking/prioritisation purposes. Illustrative examples of how DART has been used are described in Worth et al (2007).

Ranking methods provide a powerful means of sorting and grouping chemicals on the basis of multiple properties (e.g. persistence, bioaccumulation and toxicity). Ranking methods are useful not only for sorting chemicals according to their relative level of ‘concern’ (i.e. for identifying trends and defining subgroups based on different levels of concern), but also for identifying different profiles of toxicological behaviour (which can also be regarded as subgroups). Thus, ranking methods provide a means of comparing chemicals in terms of both the quantitative and qualitative differences in their toxicity profiles, and consequently provide a means of performing trend analysis and subgrouping. This is very useful for ranking a set of substances on the basis of their hazard profile to either identify which are the most representative substances to test, or which chemicals should be evaluated first.

4. END APPLICATIONS – FITNESS FOR PURPOSE

Grouping methods may be nominally categorised into so-called top-down or bottom-up approaches depending on the context of application, the available information and the types of substances under consideration. Figure 1 illustrates the concept of top down and bottom-up approaches. The context of application could be as wide-ranging as ranking/prioritisation strategies for R&D pipelines, profiling for persistence, bioaccumulation and toxicity (PBT), or more involved such as category/read-across evaluations for regulatory applications such as REACH where risk characterisation and classification and labelling are core elements. The context of use informs both the practical strategy of how a grouping is undertaken and the level of (un)certainty that is considered fit for purpose.

Figure 1: Conceptual illustration of top-down and bottom-up approaches



Chapters 4.1 and 4.2 focus on approaches that are appropriate for discrete organic substances in particular.

4.1 Top-down: screening/prioritisation

Top-down approaches can be characterised into 2 types – unsupervised and supervised though the sorts of tools and techniques are in many respects common to both.

Unsupervised

The scenario in a top-down approach is that a large dataset or inventory of substances needs to be 'grouped' into pragmatic yet manageable sizes for subsequent more detailed evaluation whether that is hazard assessment or strategic experimental testing. From a practical perspective, this would entail gathering a list of substances and 'grouping' them on some tenet of similarity. The similarity context could be purely

empirical and based on structure alone or focused towards a plausible MOA by incorporating information about a particular endpoint e.g. using specific SAR information or descriptors from an endpoint-specific (Q)SAR model. If the only information available is on the substances themselves and what can be readily computed, the grouping would be termed 'unsupervised'.

In this situation, the starting dataset or inventory of chemicals could be characterised by either chemical parameters (such as geometrical, topological, structural, physicochemical, electronic descriptors) or through fingerprints/structural features. In essence, the substances are converted into numeric descriptors that lend themselves to data-mining techniques.

There are a number of statistical approaches that can be applied to split the dataset/inventory. It is beyond the scope of this report to detail all the various data approaches available but examples include principal components analysis (PCA), clustering and self-organising maps (SOMs) as discussed in Patlewicz et al (2007). Stenberg et al (2009) exemplified how PCA could be used to select chemicals of potential concern whereas Rännar and Andersson (2010) presented an approach using hierarchical clustering to select chemicals for an environmental impact assessment.

PCA for instance is particularly useful in exploratory analysis enabling high dimensionality data to be readily visualised. Thus, it is possible to identify clusters of similar chemicals and whether these provide some insights into differing mechanisms of action. Clustering can be described as 'the process of organising objects (chemicals) into groups whose members are similar in some way', i.e. a cluster is a collection of objects (chemicals) which are 'similar' between themselves and are 'dissimilar' to the objects belonging to other clusters. A SOM is a learning algorithm which represents high-dimensional data in a low-dimensional form without losing any of the 'essence' of the data. The data are organised on the basis of similarity by putting entities geometrically close to each other. The main goal of the neural network is to map compounds from a n-dimensional into 2-dimensional space. Compounds similar in the original multidimensional space are close to each other in the map. More information about the use of neural network approaches can be found in Vracko et al (2006) and citations within.

PCA and SOM techniques are implemented in many of the standard statistical packages. Tools such as Leadscope incorporate a number of clustering approaches depending on the size of dataset (in terms of numbers of chemicals) and the sorts of results desired. In an unsupervised scenario, a typical clustering may be to extract structural clusters. Pipeline Pilot contains many different components for data mining such as clustering routines that can be derived based on structural fingerprints or on the basis of calculated molecular properties together with the other standard statistical techniques.

An example of where unsupervised approaches can be helpful is in terms of clustering/grouping substances from a (Q)SAR training set and using that as a basis of comparison to identify relevant similar analogues to substantiate a (Q)SAR prediction. For example, for a QPRF for the KOWWIN v1.68 model, a clustering was performed of the KOWWIN training set with a test set of chemicals of interest. The pragmatic structural clusters formed proved to be a useful starting point of identifying relevant analogues to substantiate the log K_{ow} predictions made for the test set chemicals (Patlewicz et al, 2011).

Aside from empirical structural grouping or through the use of numerical descriptors, SAR information can be used as a means of grouping substances. The profilers contained with the OECD (Q)SAR Toolbox, the rulebases within Toxtree or the SARs within Derek Nexus are means by which substances can be grouped on the basis of presumed MOA. For instance, substances could be grouped on the basis of chemical reaction domains pertinent to skin sensitisation such as Michael acceptors, Schiff base formers etc. (Aptula and Roberts, 2006) or grouped based on presumed acute aquatic toxicity MOA based on the OASIS or Verhaar schemes (Verhaar et al, 1992; 1995; 2000).

Supervised

In this case, some information is known about the endpoints for the inventory/dataset of chemicals whether it be predicted information from expert systems/(Q)SAR or actual experimental data and this is factored into the statistical algorithms in addition to the structural and/or descriptor information. Many of the data mining approaches highlighted already are still applicable with some nuances. For instance, within clustering techniques, the clusters are extracted to discriminate for the toxicity present. Other techniques might include recursive partitioning (encoded in e.g. Leadscope) where the aim is to find active or statistically correlated subsets based on the presence or absence of a particular combination of sub-structural features/fingerprints. It has been successfully used for molecular diversity and similarity analysis. Other techniques useful for formation of grouping incorporating activity profiles include ranking approaches. The DART tool discussed already encodes the ranking algorithms such as total order and partial order schemes. User-specific weighted scoring schemes can also be derived to integrate information from many different sources and used as a means to rank order and prioritise substances. The US EPA's ToxPi scheme is one such example of a prioritisation scheme that ranks substances on the basis of actual/predicted hazard profile with respect to endocrine disrupting effects. More information on the scheme can be found in the following reference (Reif et al, 2010). A ToxPi GUI interface has since been made available to enable users to profile-specific datasets using such a prioritisation scheme (see http://comptox.unc.edu/toxpi.php).

4.2 Bottom-up: regulatory purposes

For regulatory purposes such as where only one substance or a small handful of substances need to be evaluated and assessed, a bottom-up approach is relevant. Practically, bottom-up approaches can be either empirical or knowledge based. For the purposes of this document, they shall be referred to as analogue approaches and knowledge-based approaches.

Analogue approaches

In this case, the practical manner of forming a category or analogue approach could be through a systematic search of potential analogues using similarity indices or fragments. Examples might include web tools such as the US EPA's Analog Identification Methodology (AIM), ChemIDplus or commercial tools such as Leadscope. A number of these were described in more detail in the corresponding ECETOC report TR109 (ECETOC, 2010). A brief overview as a revision is presented in Chapter 6.1.

Knowledge-based approaches

Knowledge-based approaches encompass 'human expert rules'. Human experience for specific endpoints is often and conveniently encoded in the form of structural alerts. The types of SARs as encoded in expert systems such as those already discussed in Chapter 3.3 are a convenient means of identifying related substances that have a similar presumed MOA.

The first approach is most effective at identifying promising analogues which then need to be evaluated with respect to their relevance. This includes consideration of SAR information to provide a similarity context for the purposes of specific endpoints, a consideration of the physicochemical profile (either through the use of expert systems such as the EPI Suite tool or others) as well as a potential for activation through metabolism or other chemical transformation (oxidation, hydrolysis). The knowledge-based approach is useful for identifying and to an extent evaluation of the validity and relevance of the analogues for specific endpoints. This latter approach mimics the workflow within the OECD (Q)SAR Toolbox as far as gathering analogues together that share common profiling outcomes.

4.3 Top-down/Bottom-up: special class considerations

As described in the OECD Guidance on Grouping of Chemicals (2007a), UVCB substances generally i) contain numerous chemicals and cannot be represented by a simple chemical structure, or defined by a specific molecular formula; ii) are not intentional mixtures of chemicals; iii) many are of natural origin, including crude oil, coal, and plant extracts, and cannot be separated into individual constituent chemical species; iv) are produced according to a performance specification related to physicochemical properties (e.g. boiling point range). Important areas for consideration, and regarding which guidance at the current time is still under development, include UVCB test substance composition, characterisation of UVCBs for application of computational models, and how to define when UVCB substances are the same (OECD, 2012a).

The same general rules for the generation of non-testing data via grouping approaches (read-across and category formation) or (Q)SAR also apply to UVCB substances. Some of the current approaches for grouping UVCBs include composition (cut-off ranges, known/generic composition) chromatographic fingerprints, reference to standards, information on the production process, genus/species of origin of natural complex substances, and potentially marker chemicals (ECHA REACH TGD). If adequate information is available, properties of components may be used to determine a series of representative structures for the UVCB substance/category and these may be used for hazard assessment. In general, analytical characterisation can only specify broad classes of compounds, from which it is possible to postulate theoretical representative structures. This may be done based on existing industry knowledge (e.g. Quann, 1998), and expertise in manual and computational development of such structures.

At the time of the preparation of this report, a tool employing the Hydrocarbon Block Method, whereby a complex substance is divided into representative blocks of constituents with similar physicochemical, fate and hazard properties which can be used for risk assessment, has been developed (PETROTOX, 2011). Another effort to perform characterisation, chemical representation and modelling of UVCB substances is

underway (ECHA, 2011). Results are not currently available, although the importance of such initiatives is clearly recognised.

The creation of groups and sub-groups of UVCBs allows for determination of the intrinsic hazard properties through the application of test data, read-across, and marker substances (e.g. benzene, toluene, ethylbenzene, and xylenes (BTEX); 1,3-butadiene with specific effects as mentioned above) are known to contribute specifically to toxicity (Simpson and Comber, 2006).

Sub-types of UVCBs include (OECD, 2012a):

- Plant and animal products
  - Fragrance chemicals
  - Oligomeric/polymeric UVCBs
- Reaction products/products from industrial processes
  - Petroleum substances
  - Hydrocarbon solvents
  - Oligomeric/polymeric UVCBs
  - Inorganic UVCBs
  - Oleochemicals
  - Distillation bottoms

By nature, these substances are variable in composition. For example, petroleum products, which can be based on boiling point ranges, can have similar technical performance/specifications and yet exhibit a different composition based on the performance of catalysts, or the input of the crude oil that is being refined. A similar principal can be applied to coal-derived complex substances. The composition of natural or plant-derived complex substances (e.g. resin, some fragrance chemicals), are dependent on the refinement process, the species used for extraction, and the specific part of the organism/plant used for extraction, as well as growing conditions and maturity of the crop used for extraction.

A key point of the current discussion on UVCB substances is about composition. In order to determine the viability of using read-across one needs to understand the components of these products in sufficient detail. It is also necessary to determine which of these components are likely to drive potential effects (e.g. BTEX, 1,3-butadiene, PAHs). It is recognised that generic criteria to describe the composition of UVCBs still need to be developed (OECD, 2012a). Criteria under development should consider concentration range and typical concentration of components of the UVCB, what are the generic constituents, what are the specific constituents, how to differentiate well-defined substances from UVCBs, what are acceptable constituent concentration ranges, and how to handle substances which are difficult to analyse in practice. For many UVCB substances, standard industry methods, including spectroscopic techniques (UV spectroscopy, IR spectroscopy, NMR, and mass spectrometry) and chromatographic techniques (gas, and liquid chromatography) that may provide adequate compositional information for some of the less complex UVCBs (e.g. hundreds of components) (Concawe, 2012). For the more complex UVCBs (thousands to hundreds of thousands of components), there exist state-of-the-art techniques including two-dimensional gas chromatography (GCxGC), which can provide significant compositional insight into the substance. However, as is common with emerging technology, a high amount of effort and expertise is required to develop

methodology, and also importantly to interpret the results (Concawe, 2012). As such, methodology does not currently exist for the analysis of complex UVCBs in a standardised manner.

Current recommendations are generic and include the use of standard industry methods for characterising UVCB substances of all sub-types, to allow for a structured analytical approach that allows for accurate hazard assessment. This discussion is still on-going at the time of the preparation of this report.

5. CONSIDERATIONS FOR THE USE OF NON-TESTING APPROACHES

Regulations, such as REACH (EC, 2006), mandate that vertebrate animal testing should be conducted only as a last resort. However, it should be recognised that it is not always possible or appropriate to utilise non-testing approaches in place of animal testing. Therefore, when deciding whether or not to use read-across or other non-testing approaches the first consideration must always be whether such approaches are scientifically plausible. Specifically, does the use of these approaches permit an accurate and credible assessment of the hazards for the substance in question?

Whether submitting a dossier to a regulator or satisfying internal product stewardship needs, if the answer to this question is ‘no' then more traditional approaches encompassing a combination of *in vitro* and *in vivo* toxicity testing should be considered. If it is determined that non-testing approaches or read-across from one or more substance to another can be exploited, then a number of other factors should be taken into account when constructing the justification. For instance, in addition to determining the hypothesis supporting the grouping of substances, or the arguments for using a (Q)SAR tool to address a data gap, it is important to understand that although there are benefits to using read-across and non-testing approaches, there are also risks and implications.

The benefits are fairly clear and probably the three most apparent are:

- Reduce animal testing – legal and ‘reputational' obligation to avoid ‘unnecessary' testing in animals; avoiding animal testing due to legal restrictions (e.g. Cosmetics Directive).
- Time – non-testing approaches and read-across take less time to implement – particularly if using these approaches to characterise endpoints such as repeated-dose toxicity and reproductive/developmental toxicity.
- Money – toxicity tests are expensive. By utilising non-testing and read-across approaches multiple substances can be ‘hazard characterised' for far less money than running full toxicological studies on every substance.

It is clear that the primary driver for using non-testing approaches is the desire to minimise animal testing. However, there are many situations where the use of (Q)SAR predictions or read-across has been used to address endpoints that require no animal testing, for example, *in vitro* genotoxicity, biodegradation, toxicity to aquatic plants or microorganisms. As such, if one assumes that the justification for using read-across or (Q)SAR is valid, then the only benefits for these situations are time and money. Therefore, in a regulation such as REACH, which mandates specific data requirements that must be met in support of a registration, the use of ‘non-testing' approaches and read-across can impart some significant benefits, not limited to just the completion of a dataset without having to resort to new testing, in particular, new testing utilising animals.

It is important to note that for regulations such as REACH, at this point, registrants must have legitimate access to refer to the data they require to support their registration. As such, where an analogue or category has been used the registrant would need to purchase access to all the data necessary to support the registration dossier. Doing so may involve significant up-front costs. However, in the majority of cases this

cost are lower than that associated with performing a new set of studies, and clearly avoids conduct of new studies.

The number of data gaps that need to be filled may in fact drive whether the non-testing approach applied needs to be as extensive as a category approach which would be more incumbent on legitimate access to a wealth of data or could be more readily addressed by one or more (Q)SAR. Obviously, the latter depends on the availability of actual valid (Q)SAR models that can applied and the level of granularity they provide in terms of their predictions. Some (Q)SAR may only be capable of hazard identification whereas others might provide a quantitative measure of toxicity to permit a risk assessment. Addressing a single data gap such as an *in vitro* genotoxicity measure would not necessarily invoke a full category approach, a (Q)SAR model might be sufficient for use as a replacement. On the other hand, a 90-day repeated-dose toxicity endpoint might benefit from a category approach to demonstrate consistency in effects across a range of endpoints and thus provide a WoE approach to address such a complex endpoint. In any case, the number of data gaps and the approach to addressing them should be fit for purpose.

Having considered some benefits, it is important to recognise the risks and implications associated with utilising non-testing approaches and read-across, since acknowledging these may shape the strategy taken to justify their use.

Two of the most obvious risks and their implications are that the read-across approach is rejected, or that in using read-across or non-testing approaches, the hazards of the substance are mis-characterised – i.e. by either being too conservative (over-classifying) or not being sufficiently conservative. The rejection of a read-across approach is clearly a major concern for industry since the outcome could lead to significant additional testing required in order to 'fill the identified gaps' following rejection of read-across. Alternatively, if the use of this approach mis-characterises the hazards of the substance by not being sufficiently conservative, then workers, consumers or the environment may be placed at increased risk unknowingly. This is likely the primary reason why regulatory bodies scrutinise the use of read-across and non-testing approaches very closely.

It is indicated above that for the purposes of a REACH registration, making use of read-across can involve the purchase of access to data on analogous substances in order to support the registration dossier. If a read-across approach is then rejected then it could be considered that the time and money spent developing the read-across justification and purchasing access to data has essentially been wasted.

These risks drive the need to make sure that when read-across and non-testing approaches are utilised, they are well supported with sound and robust justification. Thus, in order to benefit from utilising read-across and non-testing approaches, it is vital that sufficient time, effort and expertise are invested in order to ensure that where these approaches are employed, they are done so in a scientifically rigorous and supportable fashion. This increases the likelihood of regulatory acceptance as well as reduce the possibility that hazards are mis-classified (for better or for worse) and that resources are wasted.

From a practical perspective, a number of additional factors can be considered such as whether the substance under study is already a member of an existing regulatory category or could be rationalised as a potential member of an existing category. The latter can have potential implications since the overall validity

of the category needs to be re-evaluated in light of an additional member. There are many tools and approaches to help in the identification of analogues (see Chapter 6 for a discussion of these) but identifying source analogues with available relevant data is not a trivial undertaking. Moreover, even if those analogues do have relevant data, the question remains whether the data is of sufficient quality to be used, i.e. are the Klimisch criteria (Klimisch et al, 1997) satisfactory for the intended purpose or are the data subject to too many additional assessment factors to account for the uncertainty that a risk assessment could not demonstrate safe use. In such a case arguably there would be merit in performing the necessary experimental testing on the target substance itself (if legally possible) to minimise the uncertainties and arrive at a more reasonable risk assessment.

6. SYSTEMATIC WORKFLOW

This chapter provides a systematic workflow of the different steps that are involved in forming a category/analogue. Identifying potential analogue(s) and evaluating their relevance for a given endpoint address the first two steps. It then discusses what the underlying rationale(s) might be for forming the category/analogue approach, the scope of the category/analogue – whether it be restricted to certain endpoints, mammalian or environmental effects, and proposes how a read-across might be substantiated for different endpoints in turn. Other aspects discussed in brief are considerations around classification and labelling of the category members or their impurities.

6.1 Analogue identification

Analogue identification is a first step when undertaking a grouping approach. Chapter 4 discussed the different end applications for grouping and introduced terminology such as top-down and bottom-up to highlight those cases where the starting point can differ and which merits a difference in approaches based on the available information and the decision making to be applied. In the case of top-down approaches, analogue identification is not really a criterion since *a priori* the analogues of interest are predetermined due to the inventory of chemicals to be grouped. For top-down approaches, the evaluation of analogues is a critical aspect.

For bottom-up approaches where the starting point is a single chemical under evaluation, identification of analogues and evaluating their relevance with respect to the endpoint/s of interest is fundamental. Depending on the end application, the strategy of analogue identification can vary considerably. For example, the analogue identification scope might be restricted to a given endpoint, or the analogue search might be more broadly undertaken to identify structurally-related analogues from which a filtering and more extensive evaluation may be conducted. A search might be performed to capture a wide selection of analogues with pockets of relevant data from which a grouping/category is attempted or the search might be restricted to substances only manufactured by a given organisation to restrict the scope of regulatory submission[1].

In either approach, it is implicit that experimental data are associated with the analogues identified and that these data are of high quality. A selection of available tools which assist in identifying analogues and evaluating their relevance with respect to their associated data are described in brief.

To date, many examples under REACH are thought to be analogue approaches highlighting a reluctance to broaden the scope to more than a handful of substances to manage the information and data that needs to be submitted in support of a substance and ensure that the relevant study summaries and access to the data usage is undertaken in an appropriate manner. For regulatory purposes, such as REACH or Classification and

[1] It is recognised that restricting the scope in this manner can result in missed analogues with relevant data. That is not to say that these analogues should not be discussed in the context of the endpoint justifications, indeed such analogues provide useful supporting information to substantiate a trend or hypothesis even if they are not included as members of the category being submitted for registration.

Labelling under the Globally Harmonised System (GHS), the quality of the analogues and the available data to be used for read-across is paramount. Data quality is obviously an issue for any evaluation to ensure that sound reasoned justifications are made, but in the case of screening exercises, there is greater flexibility to weigh and evaluate data that provide some evidence of effects, or lack of, to provide a robust assessment without the burden of representing the available data in the appropriate documented format. In these cases, there is greater scope to exploit read-across approaches without the constraints of their application under regulatory frameworks.

Hence, in the case of regulatory applications, it may be that *a priori* the substance(s) to be registered and the analogues to be used in support of the registration are already defined. In cases where there is no presumption/restriction of what analogues to use, one can rely on a myriad of tools and techniques to assist and facilitate the identification of analogues. These have been discussed in part in the ECETOC Technical Report 109 (ECETOC, 2010), and here a brief recap by way of a summary is provided. Some of these tools facilitate the identification of analogues with and without data, whereas others can be searched to find associated data on a substance by substance basis.

The most common analogue identification approaches still rely on structural similarity or sub-structural assessment. It is well established now that structural similarity is only one criterion to identifying and evaluating analogues for their suitability for read-across. Nevertheless, structural similarity provides a good pragmatic first step to identify promising analogues that could be expected to exhibit similarity in activity.

The most commonly used structural similarity approach takes the form of a similarity index, a quantitative measure between 0 and 1 which summarises the commonality in structure based on the presence and absence of particular structural fragments. By far the most common that is seen is the Tanimoto index which is defined as follows.

T = NAB / NA + NB - NAB

where NA is number of features (ON bits) in A, NB is the number of features (ON bits) in B, and NAB is the number of features (ON bits) common to both A and B

Essentially a Tanimoto index of 1 indicates the same structure, whereas an index close to 0 obviously indicates a complete dissimilarity.

Tools such as ChemIDplus, available as a service on the National Library of Medicine website (http://chem.sis.nlm.nih.gov/chemidplus) and freely available, provides the means to search extensive inventories of chemicals many of which contain links to available databases or literature information. The interface enables a user to search on the basis of substructure or similarity and provides a means of adjusting the similarity index. Results are presented as a list but need to be individually reviewed and links to databases or literature references presented if available also need to be evaluated on a case by case basis.

Scifinder (http://www.cas.org/products/sfacad/index.html), a commercial CAS registry system also enables sub-structural and similarity searches to be performed. The results also need to be reviewed individually but

the information can be saved and reviewed as and when is required which for the end user provides some greater flexibility. Literature information may be provided as part of the search results.

Leadscope (http://www.leadscope.com) is another commercial tool but herein a sub-structural or similarity search may be performed but restricted to present results for only those analogues that actually possess associated information that might be useful for read-across purposes. The results can be saved in projects for subsequent analysis but the hit list can also be exported together with the summarised data in an excel format. This is helpful in terms of being able to view a data matrix in one overview to evaluate trends. For larger hit lists, the capabilities within Leadscope's extensive data mining tools, analysing the available analogues by formulating hypothesis makes the evaluation piece of judging the relevance of the analogues identified a significantly more manageable task. Much of the data available in Leadscope has been extensively curated and for many endpoints, notable examples include genotoxicity, repro-/developmental toxicity, irritation and other. The data have been structured into templates to facilitate the search and retrieval of specific outcomes within studies. Databases include RTECS, specific FDA curated databases, NTP, and other databases such as CCRIS which are also available through ChemIDPlus.

There are other searching tools such as:

Chemfinder (http://www.chemfinder.com/chembiofinder/Forms/Home/ContentArea/Home.aspx), Chemspider (http://www.chemspider.com) and Pubchem (http://pubchem.ncbi.nlm.nih.gov/search) that allow the search of analogues.

AIM is the US EPA's Analog Identification Methodology. This tool, an interface freely available from the EPA website (http://www.epa.gov/oppt/sf/tools/aim.htm) works on a different basis. Rather than use a scoring scheme such as a Tanimoto index, the set of fragments and structural features that are encoded in the programs that EPA already uses as part of its estimation toolbox, are used as a means of identifying similar analogues but with associated data. In this case, a hit list of promising analogues is provided but the hit lists tend to be significantly smaller as only those substances that have some reference to available data are provided. In addition, with the absence of a quantitative index as a score of similarity, the user is left to judge and evaluate the analogues on an individual basis. The outputs from AIM can be exported in tabular fashion which provides a readily available excel data matrix of analogues and references to data which then needs to be sourced separately. However, at the time of writing the AIM methodology is unavailable.

US EPA are developing a system called ChemACE (Chemical Assessment Clustering Engine), a new tool to instantly ‘cluster’ all chemicals in a large user-defined inventory in a single run using structural characteristics. This would assist in the formation of groupings from a top-down approach. ChemACE is still under development and is being made available on a restricted site for beta testing and comment (http://www.epa.gov/oppt/sf/tools/chemACE.pdf).

US EPA also have invested much effort into the system ACToR (http://actor.epa.gov /actor/faces/ACToRHome.jsp) - a collection of databases collated or developed by the US EPA National Center for Computational Toxicology (NCCT). It aggregates data from over 500 public sources on over 500,000 environmental chemicals. It is searchable by chemical name, other identifiers, and by chemical structure. The data includes chemical structure, physicochemical values, *in vitro* assay data and *in vivo*

toxicology data. Chemicals include, but are not limited to, high and medium production volume industrial chemicals, pesticides (active and inert ingredients), and potential ground and drinking water contaminants.

Perhaps one of the most extensively used tools now is the OECD (Q)SAR Toolbox (OECD, 2009a). Conveniently packaged with inventories of chemicals and a number of different databases, it provides a means of identifying analogues from many sources and with available data. The current version is 2.3 (http://www.oecd.org/document/54/0,3746,en_2649_34379_42923638_1_1_1_1,00.html#Download_qsar_application_toolbox) and contains many new functionalities and capabilities including export/import capabilities to IUCLID which is inevitably helpful for REACH purposes. The current version also includes connections to other third party software which enables predictions to be generated by other tools by being run in the background. There are different means to identify analogues, from structural similarity approaches based on fingerprints to identifying starting sets of analogues with commonality in one of the many profilers within the Toolbox. For example, a substance may be profiled on the basis of different rulebases such as alerting groups for mutagenicity or could be profiled to identify the structural fragments or functional groups or chemical classes. This enables a search to be performed to retrieve analogues that might be more general in nature (structural similarity) or analogues that might be more specific to an endpoint of concern (mutagenicity).

Other tools available to help search and identify promising analogues include Discovery Gate software available from Accelrys (http://accelrys.com/products/databases/database-access/discovery-gate.html).

Identifying analogues that might be useful for UVCBs such as multi-constituents in particular is under development. Work within the OECD (Q)SAR Toolbox effort are exploring means of handling mixtures or substances with variable composition, e.g. variable chain length etc. Efforts include how to represent such substances chemically and be able to enumerate the substances into their likely combinations and permutations for evaluation in the same manner as described already.

A table summarising the tools mentioned above is provided below.

Table 1: Selected tools for the identification of analogues

Name	Link for further information
Chemspider	http://www.chemspider.com/
Chemfinder	http://www.chemfinder.com/chembiofinder/Forms/Home/ContentArea/Home.aspx
ChemIDplus	http://chem.sis.nlm.nih.gov/chemidplus/
Pubchem	http://pubchem.ncbi.nlm.nih.gov/search/
AIM	Unavailable
ACToR	http://actor.epa.gov/actor/faces/ACToRHome.jsp
OECD (Q)SAR Toolbox	http://www.oecd.org/document/54/0,3746,en_2649_34379_42923638_1_1_1_1,00.html
Leadscope	http://www.leadscope.com/
Scifinder	http://www.cas.org/products/sfacad/index.html
DiscoveryGate	http://accelrys.com/products/databases/database-access/discovery-gate.html

The majority of the tools described above also provide available data associated with the analogues identified. A number of tools and databases are available for which structure searching is not yet a functionality. Some of these are described in the European Centre for the Validation of Alternative Methods (ECVAM) search guide (Roi and Grune, 2011). Of particular note is the OECD's eChemPortal (http://www.echemportal.org/echemportal/page.action?pageID=0) which allows simultaneous searching of reports and datasets by chemical name and number, and by chemical property. Direct links to collections of chemical hazard and risk information prepared for government chemical review programmes at national, regional and international levels are provided. Classification results according to national/regional hazard classification schemes or to the Globally Harmonised System of Classification and Labelling of Chemicals (GHS) are also provided when available. In addition, eChemPortal provides also exposure and use information on chemicals. eChemPortal is akin to a 'google' for a cluster of different databases. It includes amongst others the ACToR database, ESIS, OECD specific databases as well as some of the databases that form part of the cluster in ChemIDPlus.

ESIS, the European chemical Substances Information System is a complex, heterogeneous system providing information on chemicals. It is hosted and managed by the JRC though some of the databases have been since taken over by ECHA. It includes various databases such as EINECS (European inventory of existing commercial chemical substances), ELINCS (European list of notified chemical substances), IUCLID Chemical Data Sheets, Priority Lists, Risk Assessment process and tracking system in relation to Council Regulation (EEC) No 793/93 also known as Existing Substances Regulation (ESR).

The ECHA dissemination website (http://echa.europa.eu/web/guest/information-on-chemicals/registered-substances) provides information on registered substances. This information is provided by companies in their registration dossiers. A variety of information such as hazard profile, classification and labelling is available. The amount of information provided can vary for different substances depending on the production volume of the substance. ECHA does not verify the information before dissemination. It is also available through the OECD's eChemPortal. As of 28th June 2012, the database contains 5,386 unique substances and information from 27,040 dossiers.

There are many resources available to find data. Cefic LRI has funded efforts to result in high quality databases (http://www.cefic-lri.org/lri-toolbox), EU projects such as SEURAT's COSMOS project engaged in developing toxicological databases for repeated-dose toxicity (http://www.cosmostox.eu/what/databases/), or the OSIRIS programme (http://www.ufz.de/osiris/index.php?en=18585) included development of databases for specific endpoints that were subsequently loaded into the ChemProp tool (see http://www.ufz.de/index.php?en=6738).

It is beyond the scope of this report to highlight all data sources, suffice to say that access to high quality data for analogues under evaluation is a critical step in formulating a read-across justification. A selection of common database sources is listed below.

Table 2: List of selected databases or tools containing databases

Name	Further information	Availability
OECD Toolbox	Various databases covering certain mammalian toxicity endpoints in addition to physicochemical data http://www.oecd.org/chemicalsafety/assessmentofchemicals/theoecdqsartoolbox.htm	Freely available
ECHA Dissemination website	http://echa.europa.eu/web/guest/information-on-chemicals/registered-substances	Freely accessible
eChemPortal	http://www.echemportal.org/echemportal/page.action?pageID=0	Freely available
ACToR	http://actor.epa.gov/actor/faces/ACToRHome.jsp	Freely available
ESIS	http://esis.jrc.ec.europa.eu/	Freely available
Cefic LRI	FeDTex Fertility and Developmental Toxicity in Experimental Animals RepDose Relational database on repeated dose toxicity	Freely available
EU Projects	OSIRIS: http://www.ufz.de/osiris/index.php?en=18585 SEURAT's COSMOS: http://www.cosmostox.eu/about/seurat/	
Accelrys Toxicity Database	http://accelrys.com/products/databases/bioactivity/toxicity.html	Commercial
Leadscope	http://www.leadscope.com	Commercial
LHASA Ltd's Vitic Nexus	https://www.lhasalimited.org/vitic_nexus/	Commercial

6.2 Rationale for grouping: analogue evaluation

Once potential analogues have been identified using one or more of the approaches discussed in the previous chapter, a critical next step is to determine their suitability for the specific purpose in mind. As already alluded to in Chapter 2, current regulatory guidance provides rationales that can be used as the underlying hypothesis to support a category or analogue approach. These rationales provide a convenient framework to begin an evaluation of the analogues under consideration. The rationales are namely:

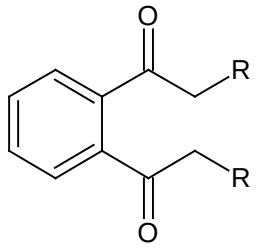
- common functional group(s) (e.g. aldehyde, epoxide, ester, specific metal ion);
- an incremental and constant change across the category (e.g. a chain-length category), often observed in physicochemical properties, e.g. boiling point range;
- common constituents or chemical classes, similar carbon range numbers. This is frequently the case with complex substances often known as substances of Unknown or Variable composition, Complex reaction products or Biological material (UVCB);
- the likelihood of common precursors and/or breakdown products, via physical or biological processes, which result in structurally similar chemicals (e.g. the metabolic pathway approach of examining related chemicals such as acid/ester/salt).

Whilst a category/analogue hypothesis may principally make reference to one of these 'similarity' rationales, in practice endpoint justifications and supporting information are multifaceted. Arguably multiple justifications serve to increase the overall confidence in the category/analogue approach.

6.2.1 Common functional group(s)

Common functional group(s) can be likened to chemical class categories. Many examples of such categories are documented in the OECD HPV programme. Examples include the ethylene glycols category, the high molecular weight phthalate esters (HMWPE) category and the acid chlorides category. In the ethylene glycols case, the hypothesis was based on the fact that the members were represented by a generic molecular structure (HO(CH2CH2O)nH where n = 1-5. The structure was such that there were two terminal hydroxyl groups and where the only variation was in the number of oxyethylene units. In the acid chlorides case, members were selected based on the commonality in molecular structure. Substances in the HMWPE were represented by the same basis structure shown in Figure 2 but with different alkyl groups (as shown by R).

Figure 2: Representative structure for the HMWPE



Whilst the members of these categories could have readily been selected using any one of the analogue identification approaches, the substantiation of a hypothesis needs to bring together a number of cross-cutting issues. These are reflected in the OECD HPV categories referenced above. Here, several general considerations are drawn out that need to be factored into any category/analogue justification and highlight the tools and approaches that can facilitate this step. Endpoint-specific justifications are discussed in more detail in Chapter 6.5.

For a common functional group or chemical class, the assumption is that only a single functional group is under consideration, i.e. a substance is categorised as an aldehyde, acid chloride etc. SAR profiling rulebases such as those encoded in Toxtree or Derek Nexus can be helpful in filtering substances that possess additional functionalities that are likely to significantly impact the activity profile. Some of the profilers within the OECD (Q)SAR Toolbox are of particular note. The ECOSAR class or organic functional groups are helpful to strictly limit substances to a particular chemical class. General mechanistic rulebases such as the DNA Binding or Protein Binding profilers are also helpful to evaluate impact of additional functional groups by judging whether these meet particular exclusion or inclusion conditions of any alerting group triggered. This step of an evaluation could be likened to the ‘structural similarity’ assessment discussed in Wu et al (2010). It is also important to note departures in a behaviour from the category which may indicate factors other than the initial commonality of functional group having an effect, for example on an expressed toxicological endpoint, and further that the same common moiety may not be relevant to all endpoints.

Impurities and purity profiles are another consideration. Particular levels of impurities may trigger classification and labelling consequences. This is discussed in more detail in Chapter 6.3.

Physicochemical similarity is important to help gauge comparable bioavailability and excretion of analogues under consideration. Parameters such as log K_{ow}, log K_{oc}, water solubility, molecular weight and vapour pressure provide pertinent information of likely environmental fate (e.g. air, water, soil compartments) or partitioning behaviour through biological membranes. Some of these parameters lend themselves to estimations – the EPISuite tools discussed in Chapter 3.3 contain a number of models that can help evaluate physicochemical similarity.

Reactivity is characterised by standard organic chemistry principles that are documented in many standard reference texts. Online sources such as the Virtual Chemistry Text book (http://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/intro1.htm) can provide general insights for standard functional groups. Certain endpoints such as skin sensitisation and genotoxicity are characterised by covalent binding as a rate determining step or MIE. In these cases, organic reaction chemistry principles such as those published by Aptula and Roberts (2006) showed that it was possible to rationalise sensitisation potential and potency with reactivity. Alerts as encoded in Toxtree, TIMES and the OECD (Q)SAR Toolbox also serve to characterise reactivity with respect to specific endpoints. Whilst some parameters such as quantum chemical parameters may be helpful to quantify reactivity (see Roberts et al, 2008), *in vitro* or *in chemico* approaches such as those published by Schultz et al (2006), Gerberick et al (2004) or Natsch et al (2007) and discussed in Schwöbel et al (2011) provide an efficient means of generating experimental bridging data, i.e. non-endpoint data but relevant to substantiate a consistent reactivity profile. Hydrolysis may be another consideration – ester groups are not necessarily electrophilic (unless activated, as is the case with phenyl esters) but they typically hydrolyse either abiotically or through metabolism. Hydrowin, a model within the EPISuite provides some information on chemical classes that are likely susceptible to hydrolysis together with their rates. Catalogic also has specific rules for hydrolysis. Work is in development for a hydrolysis profiler for the OECD (Q)SAR Toolbox. A hydrolysis simulator is available in the beta version 3 of the OECD Toolbox. Some hydrolysis rules are encoded into Toxtree as part of the Cramer and CRAFT rulebases.

Metabolism similarity is a final general consideration. Metabolic similarity is critical to demonstrate that all analogues under consideration follow the same metabolic pathway and with comparable rates. This is an important factor to consider in case exceptional metabolites are formed which result in different MOAs for the toxicity that the analogues under consideration are likely to exhibit. An example could be hexane and pentane – at face value these are structurally related, have comparable physicochemical properties and neutral organic properties, but hexane can be metabolised to hexan-2,5-dione which defines its toxicity profile; thus, read-across would be invalid between hexane and pentane. Tools, such as the profilers within the OECD (Q)SAR Toolbox, METEOR, META, TIMES or Catalogic, are useful to identify potential metabolites and the pathways by which they are formed. Tools like DiscoveryGate or LMC's Metapath provide documented experimental metabolic pathways. These provide useful information to substantiate the metabolism similarity. *In vitro* and *in vivo* toxicokinetic assays provide further information. These are discussed in more detail as part of Chapter 6.2.5.

6.2.2 Incremental and constant change across the category

The considerations to note for the rationale 'incremental and constant change across the category (e.g. a chain-length category)' are shared with those for the common functional group. The key for this rationale is

to demonstrate a trend across the category members for a range of different endpoints. Here breakpoints in a category need to be taken into account especially where log K_{ow} and water solubility thresholds are reached. These impact the potency of an effect due to bioavailability issues. For example, in a chain-length category of alpha olefins, there was a general increase in acute aquatic toxicity but at chain length of C8-C10, a cut off was reached and no additional toxicity was observed. Sanderson et al (2009) also noted a breakpoint in acute aquatic toxicity with an evaluation of long-chain alcohols. In Patlewicz et al (2001), a series of cinnamic aldehydes was investigated for their sensitisation potency. Reactivity remained constant but potency was found to be correlated to log K_{ow} which in turn was related to the chain length of the alkyl group. As the alkyl chain increased, sensitisation potency weakened. Similar effects have been noted for eye irritation of cationic surfactants, where a larger alkyl group attached to the quaternary ammonium ion resulted in less irritation (Patlewicz and El-Deredy, 1999).

6.2.3 Common constituents or chemical classes

Common constituents or chemical classes, similar carbon range numbers exemplifies UVCBs. Examples have been published in the REACH Technical Guidance as well as the REACH case studies (Worth and Patlewicz, 2007) including hydrocarbon solvents, petroleum products, coal-derived complex substances and natural complex substances. However, there still exist a number of areas for further development, particularly surrounding substance identification and composition of UVCB of all types.

6.2.4 Other considerations for analogue evaluation

In addition to the types of general considerations for evaluation of analogues, there are also a number of general factors that can help increase confidence in a category approach. Groupings with a large number of chemicals are favoured since quantitative trends can be more readily derived. This may be particularly relevant for large groups where totality of information is sparsely distributed across category members but where bridging studies such as cytoxicity or reactivity assays can be conducted. Consistency across endpoints may also help to increase confidence in a category/analogue approach, especially where MIEs are common; for example, skin sensitisation and genotoxicity are underpinned by electrophilicity (Schultz et al, 2006; Mekenyan et al, 2010).

The level of confidence required may depend on the regulatory consequences; i.e. a higher level of confidence may be necessary for demonstrating the absence of a given hazard than for demonstrating presence of the hazard. Conversely, a lower level of confidence may be sufficient for a screening purpose for prioritisation. Alternatively, the endpoint may set the confidence level, potentially CMR/PBT more so than other endpoints and different levels of confidence may be required depending on the use pattern/exposure level of the given chemicals; e.g. likely exposure of the general population vs. restricted to industrial use.

6.2.5 The metabolic pathway justification

Regulatory guidance (ECHA REACH TGD; OECD, 2007a) is reasonably comprehensive in describing how a category based on a metabolic pathway can be defined and used to support read-across from one member to another. For convenience, the term 'metabolic category' has been used throughout this report. Although it is covered in the guidance, it is worth noting that for categories utilising a metabolic-pathway justification, read-across should be restricted to assessing systemic toxicity due to the potential for significant differences between local and portal of entry effects. In addition, metabolic categories are not intended to cover a read-across between parent and downstream minor metabolites but should be limited to primary and secondary metabolites at most. This is important to ensure that a read-across is not structured to penalise substances that have no inherent issues but can form a downstream minor metabolite associated with a hazard profile of concern.

The category based on a metabolic pathway is perhaps unique amongst the other justifications for forming a category. Unlike other categories where structural similarities and similar physicochemical properties drive the category formation, leading to identification of trends in properties, the categories that utilise metabolism as a primary justification are reliant on the fact that one member becomes another, thus data on one can be used to predict the hazards of the other. At first glance, this may make it easier to build such a category, and also easier to assess its validity. However, experience with the evaluation of read-across arguments based on metabolic pathways has highlighted some key points that should be taken into consideration when building or assessing such a category.

Category size and uncertainty

The primary hypothesis that underpins a metabolic category is that Substance A is metabolised to Substance B, C, D etc. Therefore, it may be possible to use data from the parent to identify the hazards of the metabolites, or vice versa. This can limit the category to a small number of substances (typically 2-3) and the nature of the metabolic pathway has a significant influence on which of the category members can be used as data sources for read-across to the other members. The small size of the category may potentially lead to greater uncertainty when utilising read-across, particularly when the data-rich compound is the primary metabolite rather than the parent compound.

One possibility to address this is to increase the size of the category by combining the metabolic justification for forming the category with another justification such as an increase in chain length. An example of this could be a group of linear alcohols and their acetates or propionates. The basis for the read-across would be that they are all similarly metabolised, firstly with the removal of the acetic acid or propionic acid group, and then the alcohols are converted through to acids. By broadening a category in this way the trends observed within the larger group help to reduce the uncertainty when using read-across between a particular metabolic pair, such as butyl acetate and butanol.

The robustness of the metabolic justification

One of the challenges with a metabolic category is that it is utterly dependent on the metabolic data which demonstrate the pathway exists and that it is sufficiently rapid or thorough such that there is confidence the

data on the metabolite accurately characterise the hazards of the parent or vice versa. This opens this type of category to a type of scrutiny not possible for the other types of category because one must convince the reviewer that the metabolism is both present and sufficiently rapid to allow read-across between the parent and metabolites. The metabolic pathway must also be consistent between experimental animals and humans, and this is sometimes overlooked.

The regulatory guidance documents (OECD, 2007a; ECHA REACH TGD) state that where a metabolic pathway is particularly well understood and known to be present it may not be necessary to generate new data to support the read-across. Specifically they describe, *"certain metabolic processes are ubiquitous and well understood and these can be presumed to occur without performing in vivo experiments"*. However, this statement is not referenced to clarify which metabolic processes are ubiquitous. For example, the metabolism of a short-chain alcohol through the aldehyde to the acid is very well understood, and known to occur in experimental animals and humans. As such, using read-across from propanol to propanoic acid should not require *in vitro* or *in vivo* metabolism data to support this. However, in general, toxicokinetic data should be considered essential even in situations where the metabolic pathway is thought to be well known, such as hydrolysis of ester bonds to release an acid and an alcohol or ω-1 oxidation of an aliphatic hydrocarbon. The justification for this is that as indicated above, the validity of metabolic pathway categories depends not only on the existence of the pathway, but also the rate and extent of metabolism. For example, if metabolism is not sufficiently rapid or complete then the parent compound may exist in the body long enough to exert toxicity not observed with the metabolite. Conversely, if read-across is from the parent to the metabolite, then a slow rate of metabolism could mean that the body is exposed only to a low amount of the metabolite and this may result in a potential hazard of the metabolite being missed.

The difficulty in presenting a robust justification for a metabolic pathway category is therefore providing the right experimental data and argumentation to show the existence of the metabolic pathway, and then demonstrating that the rate of metabolism is fast enough to support the use of read-across.

An example of a recent case where this was pertinent is the use of read-across between a propylene glycol ether and its acetate. In this case, the data on the glycol ether were used to address several endpoints for the acetate, including repeated-dose toxicity and developmental toxicity. *In vitro* metabolic data demonstrated that the metabolism occurred, and that the half-life was between 10 and 14 minutes in rat plasma (Dow, unpublished). The critical questions in the acceptance or rejection of this read-across were therefore: was the rate at which the ester bond was hydrolysed 'rapid' enough? Would the metabolism happen in 'barrier' tissues, such that the 'predominant' exposure would be to the primary metabolite? The key words in these questions are 'rapid', 'barrier tissues' and 'predominant'. The reason these words are so important is that they are subjective and therefore open to interpretation. How rapid is rapid enough? Is it possible to derive a 'cut-off limit' for an acceptable rate of metabolism? Are the only barrier tissues the skin, lungs and the gastrointestinal tract, or can one argue that the liver, the placenta, the blood brain barrier etc. are also barrier tissues? This is particularly important when looking at specific toxicological endpoints such as developmental toxicity. What proportion of parent and metabolite in the body would allow the conclusion that the predominant exposure is to the primary metabolite? The lack of clarity regarding what is acceptable and what is not regarding these parameters means that the formulation of a read-across argument using metabolism as a justification is potentially fraught with uncertainty and particularly so if there is an inherent absence of toxicity for both the parent and metabolite.

Realistically, the only way to address this lack of clarity and the ever present uncertainty is not to attempt to define specific cut-off levels for what is rapid or predominant or to limit the definition of barrier tissues to skin, lungs and gastrointestinal tract, but rather to assess the available toxicokinetic data in the light of the entire toxicological data base in order to determine the validity of the approach.

Once the metabolic pathway has been demonstrated, if one can show that the toxicological profiles of the category members are consistent, then the use of read-across is strengthened, particularly if the category contains other structurally similar metabolic pairs or sequences. Additionally, if when looking at other, structurally similar metabolic pairs a pattern emerges regarding toxicological differences between parent and metabolite, then this can be critical in supporting the use of read-across. For example, reading across from pentanol to pentyl propionate for reproductive toxicity. Concluding that pentyl propionate is not a reproductive toxicant because pentanol and propionic acid are not, can be supported by demonstrating that butanol and butyl propionate, propanol and propyl propionate are also not reproductive toxicants and that there is no evidence that the propionate is more toxic than the alcohol. Similarly, in the example above of the glycol ether and its acetate, the use of read-across between the glycol ether and the acetate are supported by the consistency of their toxicological profiles for acute, repeated-dose and genotoxicity endpoints, and an absence of reproductive and developmental toxicity across the group of commercially available propylene glycol ethers and their acetates[2].

What information can support a metabolic category?

Data on the metabolism of the category members is clearly critical. A metabolic category that provides no support (theoretical argumentation or actual data) would be very difficult to accept. The metabolism data that can be used to support this type of category is covered in the available guidance documents and are not reiterated here. However, if the category is to be made as robust as possible, and the uncertainty associated with read-across kept to a minimum, then as indicated above, it is necessary to have more than just a hypothesis about metabolism or toxicokinetic data to support read-across within this type of category.

The more toxicological data on the category members, the better, and consideration should be given to investing in toxicity or bridging studies on each of the members to help underpin the read-across. For example, generating a combined repeated-dose toxicity and reproductive/developmental screening study on each category member may appear to be an extensive undertaking. However, if the intent is to only generate more extensive studies on one of the category members, being able to demonstrate consistency across the category is critical and limits the likelihood that these studies have to be performed on other category members. The specific choice of a repeated-dose combined with a reproductive/developmental screening study is to address the observation that certain endpoints, such as reproductive or developmental toxicity, appear to require a higher burden of proof than others. This is dealt with more specifically in the endpoint-specific guidance that follows (Chapter 6.5).

[2] A particular isomer of the propylene glycol methyl ether and dipropylene glycol methyl ether (the beta isomer) is known to be a reproductive and developmental toxicant. However, this isomer is present at a level of <0.3% of the commercially available glycol ethers.

When conducting targeted toxicity or bridging studies it is possible to modify them to minimise the use of animals, whilst not compromising the data generated. For example, conducting a 'limit dose' study on each category member with a single dose level and incorporating some toxicokinetic assessment in the study design, can demonstrate toxicological consistency or potential trends and provide the toxicokinetic data necessary to support the metabolic hypothesis behind the category.

6.2.6 Read-across considerations for metal compounds

The development of (Q)SAR methods for metals and inorganic metal compounds has not been as actively pursued as for organic substances. The basic assumption using this approach is that it is the bioavailable metal fraction that is toxicologically relevant (e.g. free metal ion or other specific metal species complexes). For simple metal salts, the bioavailability of the metal ion or complex is rather determined by the respective physiological environment than by the anionic component. However, the toxicity of the anion, which can be of inorganic or organic (e.g. chelates) nature, needs to be assessed initially in a comparative manner as well. Several methods have been developed to assess bioavailability of metal ions from more complex compounds and those that are not water soluble.

In case (eco)toxicity data are lacking for a specific metal or metal compound, read-across data from other inorganic compounds of the same metal could be considered. The assessment of (eco)toxicity for specific metal or metal compounds can be based on the observation that adverse effects are a consequence of exposure to the bioavailable metal ions, as opposed to the parent substances. The basis for this approach is that the parent substances (e.g. $NiCl_2$, $NiSO_4$, and $Ni(NO_3)_2$) all release the same toxicologically relevant metal ion (i.e. Ni^{2+}). Therefore, the toxicities of the parent substance are the same when normalised to the concentration of the free metal ion. As for toxicological considerations, bioavailability of the metal ions may need to be assessed in different environmental media and the environmental parameters that influence the availability in environmental compartments may vary from metal to metal and may also need to be considered in the exposure assessment.

In case additional testing is needed, this testing is usually done with a readily soluble salt where the toxicity of the anionic component is not likely to 'overshadow' the cation and where the bioavailability of the metal ion is high in order to mimic a worst-case scenario. For the most part, testing is generally done with chloride, sulphate, or nitrate salts.

6.3 Considerations of impurities/purities

Substance identity is a key input into the grouping of substances for the purpose of read-across. Without understanding the composition or defining characteristics of substances it is not possible to state whether they are sufficiently similar to allow them to be grouped together. In the case of mono and multi-constituent substances, it is critical to know the identity of the main constituents as these are the primary driver for grouping the substances together and likely drive the hazardous properties. However, when grouping substances one must also address any significant impurities present in the composition of the members. This is a key consideration that is addressed in the reporting formats described for both analogue and category

approaches. In most cases the presence of impurities should not prevent the grouping of substances, however where one member of the category contains an impurity that impacts the classification or contributes in some way to the toxicity profile then it should be considered whether data from this substance should be read-across to other members that do not contain such an impurity.

When assessing whether impurities may impact formation of a category or reduce the reliability of read-across, some considerations are:

- Do all members contain the same or structurally similar impurities?
- Is the hazard profile of any impurity known?
- Do any of the impurities impact the classification and labelling of the substance?
- Has toxicological test data been generated on test materials containing the impurities?

The best case is where all members contain the same or structurally similar impurities, or where the levels of impurities are low enough that they would not impact the results of toxicity tests or classification and labelling. In the other situations, care must be taken when using read-across and it is important to specifically address how impurities may impact the use of read-across for each endpoint. For example, in a category of linear alkanes ranging from heptane to nonane, n-hexane is a potential impurity. N-hexane is classified as a reproductive toxicant and the cut-off for classification is 3% (v/v); so if it were present as an impurity at >3% in any of the category members, it would lead to classification as reproductive toxicants.

In spite of its potential reproductive toxicity, the presence of n-hexane as an impurity would not affect endpoints such as acute toxicity, irritation, sensitisation and genotoxicity. Hence, if only one member has n-hexane as an impurity at >3% then data for these endpoints could still be used in read-across to the others and vice versa. For the endpoints such as repeated-dose and reproductive toxicity more consideration is necessary. In reality, n-hexane is not a potent reproductive toxicant in animal studies, with high doses being necessary to produce an effect (Demartino et al, 1987; Daughtrey et al, 1992). As such, its presence as an impurity at a level of >3% is unlikely to impact the reproductive toxicity. Therefore, data for this endpoint could also be read-across between category members, keeping in mind that regardless of the data, the classification for reproductive toxicity would still be necessary where n-hexane was present in a category member as an impurity at concentrations >3%.

In cases where impurities are present in category members but little is known of their toxicity, (Q)SAR tools such as those described elsewhere in this report can be used to give an indication as to whether these impurities could have biological activity (such as genotoxicity or sensitising potential) that could influence how read-across can be used within the category.

6.4 List of endpoints covered

The read-across within a category is dependent on an underlying rationale to justify the similarity between members for a specific endpoint of concern, i.e. an endpoint-specific category. The similarity context may differ depending on what the MOA and molecular initiating event is for the particular toxicity. Some

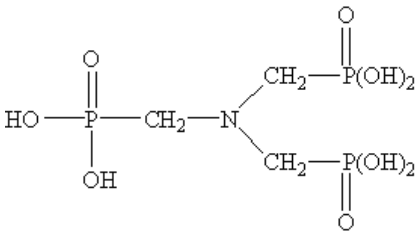
members of a category could be excluded for a given endpoint or in contrast not all endpoints might be relevant for all category members.

Here, a couple of categories are highlighted to exemplify where local effects such as skin and eye irritation may not be applicable for read-across despite other endpoints being valid and where metabolism can play a significant role.

As an example, the category of amino tris(methylenephosphonic acid) and its sodium salts has been evaluated under the OECD HPV programme, SIAM 18 (2004) [See http://webnet.oecd.org/hpv/UI/handler.axd?id=eb3605bc-0660-4915-9316-47095079fced]. These are closely-related alkyl phosphonic acids and their sodium salts. The different salts are prepared by neutralising the acid to a specific pH. The effect of the counter-ion (sodium) is assumed to be insignificant and the category members are fully dissociated in dilute aqueous solutions.

The dominant characteristic across all three categories is the presence of several phosphonic acid functions, which can ionise in aqueous solution to phosphonate anions.

Figure 3. Amino tris(methylenephosphonic acid) category



To characterise the chemical further, the backbone is considered to be the amino tris(methylenephosphonic acid) anion. All category members are highly adsorbing, highly water soluble and have similar use patterns. They all chelate metal ions so they have the potential to disrupt bioavailable concentrations of metallic cations in the blood of fish and invertebrates and to cause nutrient depletion to algae and plants by complexing trace metal cations. Direct read-across was used for most endpoints. However dermal and eye irritation studies were considered separately for the acid and their salts. Acids and their salts may give rise to differences arising from pH effects such that skin and eye irritation are likely to be different for an acid compared with its salt.

In this instance, the effect of the counter-ion, i.e. the Na-ions, was considered insignificant and did not need be taken into account in the assessment, because the exposure to this chemical would result in a negligible increase in the normal background levels of these ions.

As a second example, an endpoint-specific category could be foreseen for a homologous series of alkanes for acute aquatic toxicity effects in fish, daphnid and *algae*. For aquatic toxicity, a baseline narcosis mode of action would be presumed such that a trend of increasing toxicity with log K_{ow} or chain length would be anticipated. For systemic effects, the mode of action could well differ with metabolism being a significant factor. For example, within the series of alkanes, n-hexane exhibits toxicity that is mediated by its metabolite hexane-2,5-dione. It is believed that hexane-2,5-dione reacts with the amino group of the side chain of lysine

residues in proteins, causing cross-linking and a loss of protein function. In contrast, n-pentane is hydroxylated to corresponding alcohols (2-pentanol and 3-pentanol) hence resulting in a different toxicity profile. Thus, despite the similarity in parent alkanes, differences can result from the metabolites formed and both affect the hazard profile exhibited and invalidate the read-across approach proposed.

Overall, careful evaluation of the proposed category rationale for all the endpoints under investigation define the applicability domain of the category and which endpoints are appropriate to consider for read-across.

A table of available (Q)SAR tools and the endpoints that they cover is provided here as a summary to introduce the endpoints discussed in the next chapter and to provide additional context to Chapter 3.3.

Table 3: List of key (Q)SAR tools and the endpoints that they address

Endpoint	Tool
Acute oral toxicity	T.E.S.T[1]
	TOPKAT[1]
	OECD (Q)SAR Toolbox[2]
Acute inhalation toxicity	TOPKAT[1]
	OECD (Q)SAR Toolbox[2]
Skin irritation	TOPKAT[1]
	BfR rulebase within Toxtree[3]
	BfR rulebase within OECD (Q)SAR Toolbox[3]
	Derek Nexus[4]
Eye irritation	BfR rulebase within Toxtree[3]
	BfR rulebase within OECD (Q)SAR Toolbox[3]
	TOPKAT[1]
	Derek Nexus[3]
Phototoxicity	TIMES[4]
Endocrine disruption	TIMES (Aromatase inhibition, Oestrogen, Androgen receptor-binding affinities) [4]
	OECD (Q)SAR Toolbox (Oestrogen receptor-binding affinity profiler) [3]
Skin sensitisation	CAESAR[1]
	TIMES[4]
	TOPKAT[1]
	Derek Nexus[3]
	MCASE[1]
	OECD (Q)SAR Toolbox (Protein binding profilers) [3]
	SMARTS alerts within Toxtree[3]
Mutagenicity: Ames	TOPKAT[1]
	TIMES[4]
	OECD (Q)SAR Toolbox (DNA Binding profilers) [3]
	Benigni-Bossa rulebase – Toxtree[3]
	Benigni-Bosa rulebase – OECD (Q)SAR Toolbox[3]
	MCASE[1]
	T.E.S.T[1]
	Caesar[1]

Endpoint	Tool
	Derek Nexus[3]
	Lazar[1]
Mutagenicity: *in vitro* chromosomal aberration	TIMES[4]
	Derek Nexus[3]
Carcinogenicity	TOPKAT[1]
	MCASE[1]
	Caesar[1]
	Oncologic[4]
	Lazar[1]
Developmental toxicity	Caesar[1]
	T.E.S.T[1]
	Derek Nexus[4]
	TOPKAT[1]
	MCASE[1]
Reproductive toxicity	Derek Nexus[4]
Repeated-dose toxicity	Lazar (MRDD) [1]
	TOPKAT (LOAEL, MTD) [1]
	Derek Nexus[4]
	OECD (Q)SAR Toolbox (NEDO, Fraunhofer)[3]
Acute aquatic toxicity (fish)	ECOSAR[4]
	TOPKAT[1]
	T.E.S.T[1]
	TIMES[4]
	Verhaar rulebase – Toxtree[3]
	Verhaar rulebase – OECD (Q)SAR Toolbox[3]
	OASIS MOA – OECD (Q)SAR Toolbox[3]
	Lazar[1]
Acute aquatic toxicity (daphnid)	ECOSAR[4]
	TOPKAT[1]
	TIMES[4]
	T.E.S.T[1]
Aquatic toxicity (algae)	ECOSAR[4]
	TIMES[4]
Bioaccumulation: BCF	Catalogic
	BCFBAF[4]
	Caesar[1]
	T.E.S.T[1]
Biodegradation	Biowin[4]
	TOPKAT[1]
	Catalogic
Metabolism	MetaPrint2D[5]
	METEOR[4]
	TIMES[4]
	META[5]
	OECD (Q)SAR Toolbox[4]

Endpoint	Tool
Physicochemical properties: log K_{ow}	KOWWIN[1]
	TOPKAT[1]
	CAESAR[1]
	SPARC[1]
	ACD Labs[1]

[1] Statistical model: To demonstrate consistent predictions.
[2] Data available to facilitate grouping.
[3] To indicate MOA.
[4] To provide MOA and qualitative/(semi) quantitative predictions.
[5] Statistical model: to provide qualitative predictions.

6.5 Endpoint by endpoint justification

Whilst a rationale needs to be proposed to underpin a given category, a critical component in demonstrating that the category is robust and scientifically credible lies in providing a comprehensive justification on an endpoint by endpoint basis. The decision on which and how many endpoints to fill in data gaps by non-testing methods depends on the ultimate purpose of forming the category. For regulatory purposes, e.g. REACH where a larger range of endpoints is required to be addressed, a larger base set of test data (e.g. physicochemical, basic (eco)toxicity and environmental fate) would be needed for each chemical before one can confidently decide to group chemicals into a single category. There is also higher confidence in the robustness of the category when the number of category members is not too large (as an arbitrary number, less than 10 is proposed here). Before filling in data gaps for more complex endpoints, e.g. reprotoxicity and development toxicity, one may require the existence of similar trends in test data for lower endpoints, e.g. genotoxicity and skin sensitisation. For product stewardship or screening purposes, it may be possible to be less stringent in the amount of test data required before a category can be formed. A category could potentially contain tens to hundreds of chemicals and data gaps may be filled in using (Q)SAR approaches, with testing usually reserved for a few priority compounds.

This section characterises the considerations that may come into play for the evaluation of analogues for each key endpoint within the category as outlined in Chapter 6.2, but focuses in on how those considerations are substantiated by the available experimental data. (Q)SAR information in terms of what structural/physicochemical information is pertinent for a given endpoint can provide a means to demonstrate commonality in chemical mechanisms or modes of action.

6.5.1 Physicochemical parameters

Physicochemical parameters play a critical role in addressing many aspects of a substance's behaviour, such as likely bioavailability, environmental fate and ecotoxicity of a chemical. They may also play a role in helping to characterise chemical similarity for read-across purposes. In many cases and notably for regulatory purposes, experimental values are likely to be generated. Key properties include vapour pressure, log K_{ow}, water solubility, molecular weight as well as pKa and log D for ionisable substances. Estimates can be reasonably generated for log K_{ow} for neutral discrete organics. Log K_{ow} is one of the most important

parameters since it plays a role in estimating bioaccumulation potential by way of the bioconcentration factor, sorption, acute aquatic toxicity as well as parameters such as absorption (oral or dermal in particular). Vapour pressure and water solubility are pertinent for estimating the Henry's Law constant. Water solubility is also a critical factor when evaluating other studies including aquatic toxicity and hydrolysis. Molecular weight is a key parameter for size properties – important when considering absorption or bioaccumulation. The pKa-value is known to be related to irritation/corrosion effects as well as being key to rationalising sorption studies of ionisable substances.

6.5.2 Aquatic toxicity

The modes of toxic action in ecotoxicology are have been extensively studied and as a result, there are many (Q)SAR for aquatic toxicity endpoints available. Several modes of action were identified by McKim et al (1987) and subsequently by Bradbury et al (1990). These different modes of action are distinguished experimentally in terms of fish acute toxicity syndromes (FATS), which are characterised by defined combinations of respiratory, cardiovascular, and physiological responses in fish after acute exposure to a chemical. Various workers have attempted to characterise these modes of action. McKim et al (1987) distinguished six different modes of action. Russom et al (1997) on the other hand suggested seven modes of action whereas Verhaar et al (1992) associated four modes of action with structural characteristics. This last group provided a convenient means of categorising substances according to their likely mode of action. The four modes of action were respectively: inert, less inert, reactive and specifically acting chemicals. The first 'inert' is otherwise termed 'narcotic' or 'neutral organic' in order to indicate a narcosis mode of action. Here the overall response of fish to narcotic chemicals includes a dramatic decrease of all respiratory-cardiovascular functions and classic anaesthesia effects such as loss of reaction to external stimuli, loss of equilibrium and decline in respiratory rate and medullary collapse. Since every compound in theory can act as a narcotic, this mode of action is considered the baseline effect. Hydrophobicity has been found to be a key chemical feature in determining the effects of narcotic organic compounds in aquatic systems (Donkin, 1994). The most common measure of hydrophobicity is log K_{ow}. Konemann (1981) developed the 'classic' general narcosis (Q)SAR for toxicity to *Poecilia reticulata* (guppy) which related log K_{ow} to LC_{50} (log of the molar LC_{50}). This was subsequently found to be applicable for prediction of toxicity to a range of aquatic species, not only other fish species but other organisms such as *Daphnia magna*. It is often referred to as the 'baseline toxicity equation' and compounds which fit this equation are usually referred to as general narcotics. ECOSAR v1.11 contains a variant on this general narcosis equation for fish, d*aphnia* and green algae.

Less inert or polar narcotics have been shown to be slightly more toxic than narcotics but their toxicity is still reasonably modelled using log K_{ow} albeit the slope and intercept both differ. (Q)SAR do exist for the different species that are applicable for polar narcotic substances. Examples can be found referenced in the REACH technical guidance (ECHA REACH TGD). For reactive and specific-acting chemicals, few efforts have been made to develop (Q)SAR. In the former case, of the models available, quantum chemical descriptors encoding reactivity have been utilised. ECOSAR v1.11 does not derive mode of action (Q)SARs and instead uses the derivation of chemical class-specific SARs, all relying on log K_{ow} as the input parameter.

In terms of category/analogue approaches, several strategies exist to facilitate read-across for aquatic toxicity endpoints. The Verhaar et al (1992) categorisation scheme is encoded in both Toxtree and the OECD (Q)SAR Toolbox. Other profilers such as the OASIS MOA, an abridged version of the Russom et al (1997) scheme, are also contained within the Toolbox. The ECOSAR chemical classes are also implemented in the Toolbox. These profiling rulebase schemes are thus helpful to indicate likely mode of action. Within an analogue approach, a read-across could be performed between two analogues which were demonstrated to share a common mode of action, e.g. likely toxicity values could be quantified using log K_{ow} for substances that were polar or non-polar narcotics. For substances that were shown to demonstrate reactive features, data from *in chemico* reactivity assays (such as those discussed in Chapter 6.2) might provide complementary information to substantiate a read-across. Since electrophilicity can be a key variable characterising the MIE, i.e. covalent binding information for other endpoints, including mammalian endpoints –such as mutagenicity or sensitisation– may provide some additional insight regarding reactivity potential. Other species information could also provide a WoE approach to addressing data gaps e.g. information from *daphnia* and *algae* could fill data gaps for fish. In a category approach, a trend analysis using log K_{ow} may prove helpful to interpolate missing values bearing in mind the cut offs for high log K_{ow}/low water solubility (Schäfers et al, 2009). Alternatively, a (Q)SAR-based approach could be applied to fill data gaps by demonstrating that for a presumed mode of action, the specific (Q)SAR was estimating values in good agreement with known experimental values. Given that these modes of action are so established and the available (Q)SAR more developed, an effective strategy may be to rely on a combination of (Q)SAR information utilising measured log K_{ow} together with data in other species.

A recently developed model, PETROTOX, has been developed to apply to hazard assessment of complex hydrocarbon substances; a complex hydrocarbon substance is divided into representative blocks of constituents with similar physicochemical, fate and hazard properties which can be used for risk assessment (PETROTOX, 2011). This model is based on narcosis via the target lipid model as described in several papers, and which has been found to be applicable for acute and chronic toxicity endpoints (Di Toro et al, 2000; Di Toro and McGrath, 2000; McGrath et al, 2004; 2005; Redman et al, 2007; McGrath and Di Toro, 2009; Kipka and Di Toro, 2009).

6.5.3 Biodegradation

Biodegradation is often preceded by the terms primary or ultimate. Primary biodegradation describes the initial transformation of a chemical by micro-organisms to another organic chemical, a transformation product or metabolite; ultimate biodegradation describes the mineralisation of the parent compound to inorganic endproducts and biomass (Pavan and Worth, 2008).

Tools/Databases

Qualitative information is available for a number of biodegradation pathways, most notably the University of Minnesota Biocatalysis/Biodegradation Database (http://umbbd.msi.umn.edu/). The suitability of these data for use in hazard, persistence and risk assessment needs careful consideration and may only contribute as part of a WoE assessment if other data are available. Other available sources of biodegradation data that are useful for developing *in silico* models are: (i) the BIODEG and BIOLOG databases, developed by the

US EPA and the Syracuse Research Corporation, ii) the MITI-I database (iii) the California Department of Food and Agriculture Biodegradation Database. In addition to online databases, biodegradation data have been collected in a number of books (Alexander, 1999; Pitter and Chudoba, 1990). Some of the biodegradation data from these sources has been used to develop prediction models e.g. Catalogic, TOPKAT, BIOWIN, META, Danish (Q)SAR database. More information about these models is provided in Chapter 3.3 as well as the REACH technical guidance itself (ECHA REACH TGD).

Issues for consideration

Biodegradation information is important for (i) classification and labelling, (ii) persistence determination in PBT assessment, (iii) waivers for hydrolysis and adsorption-desorption testing, and (iv) for building a WoE case for bioaccumulation testing. The ability to predict biodegradation pathways accurately has practical implications. Many companies invest significant resources to predict the biodegradation pathways of their new compounds to avoid the commercialisation of materials that are later found to have unfavourable profiles (Wackett, 1999). As such it is important that test data and predictions from Quantitative Structure-Biodegradation Relationship [(Q)SBR] models are interpreted correctly before data gap filling is conducted, and these are briefly discussed below.

Test data

When evaluating whether test data for one substance can be read across to an analogue, several factors need to be considered. Depending on the test method, a pass criterion of 60% and 70% degradation as defined in the respective guidelines should be applied (OECD TG 301, 1992). It should be noted that substances which are poorly soluble in water may cause significant difficulties in the conduct of the test. In particular, (potentially) low levels of biodegradation may be observed due to low substance availability. Verifying whether modifications to the test system were implemented in order to improve substance availability also needs to be taken into account (e.g. the use of volatile solvents or silica gel). There are provisions in REACH guidance to allow for enhancements to standard biodegradation tests for use in PBT assessment. These enhancements do not apply to classification and labelling.

Not all of the various screening tests are suitable for the testing of all types of substances, and results obtained by the use of a test procedure which is not suitable for the specific substance should be evaluated carefully before a decision on its use is taken.

In silico models

(Q)SAR for predicting ready biodegradability are continually being improved with respect to quantitative prediction of rapid degradation. At the moment, BIOWIN, MultiCASE/META and Catalogic are the most commonly used approaches for estimating the biodegradation potential of organic compounds for regulatory purposes. In general, the models are better suited for identifying non-readily biodegradable chemicals rather than readily biodegradable chemicals (ECHA website 2012).

The development of (Q)SBR has been relatively slow compared with (Q)SAR for toxicity endpoints, mainly because of the nature of the biodegradability endpoint. Biodegradation is a complex biochemical process

that depends not only on the amount and structure of the chemical, but also on environmental conditions into which the chemical is released. The modelling is further complicated by the fact that the results of the biodegradation tests are strongly influenced by the physicochemical properties of the chemical and the nature of the microorganisms in culture medium.

6.5.4 Bioaccumulation

Bioaccumulation is the process whereby the chemical concentration in the tissues of an organism increases as a result of chemical uptake through all routes of chemical exposure (e.g. dietary absorption, transport across the respiratory surface, dermal absorption). Bioaccumulation occurs under field conditions. The term 'bioconcentration' refers to the increase (or lack of) of a test substance in the tissues of an organism upon aqueous laboratory exposure (e.g. OECD TG 305, 2012d). Within the EU, a substance is considered to fulfil the bioaccumulative (B) criterion when the BCF is greater than 2000. The bioconcentration factor (BCF) in aquatic organisms is traditionally used as an initial criterion for bioaccumulation assessment, especially where consumption of contaminated food is not relevant. Consideration of bioaccumulation via dietary exposure both in the laboratory as a biomagnification factor (BMF) or inclusion of dietary exposure in field measurements as a bioaccumulation factor (BAF) can also be used in bioaccumulation assessment (ECHA REACH TGD). Field measurements of bioaccumulation in food webs may be used to determine trophic magnification factors (TMF) which may also contribute to bioaccumulation assessment (ECHA REACH TGD). A framework has been proposed recently (Gobas et al, 2009) as a scientific ground for harmonisation of bioaccumulation assessment by different regulatory agencies and includes making use of alternative testing strategies.

Issues for consideration: category formation and data gap filling

If a substance belongs to a class of chemicals that are known to accumulate in living organisms, it may have a potential to bioaccumulate. If a valid BCF for a structurally closely related substance is available, read-across may be applied. Data gaps can also be filled in using (Q)SAR models. Common tools/databases often used to estimate/provide BCF values include BCFBAF (part of EPISUITE), Catalogic, CAESAR BCF model, US EPA AQUIRE, Japan METI-NITE, Syracuse Environmental Fate Database, ESIS, Canadian Database, CEFIC LRI-BCF, and US EPA PBT profiler.

When applying (Q)SAR models, expert systems or read-across several important aspects need to be considered, especially related to aspects/properties of a chemical that may reduce or increase its bioconcentration. These include the following properties.

Hydrolysis

For substances discharged mainly to the aquatic environment, hydrolysis may significantly reduce the extent of bioconcentration in aquatic organisms, especially where the half-life, at environmentally relevant pH values (4-9) and temperature, is < 12 hours (ECHA REACH TGD). It may be more appropriate to consider the fate of the hydrolysis products instead. Catalogic contains rules to simulate hydrolysis and these will additionally be included in the OECD (Q)SAR Toolbox v3, due for release October 2012.

Adsorption

Adsorption onto biological surfaces, such as gills or skin, may also lead to bioaccumulation and uptake via the food chain (ECHA REACH TGD). Hence, high adsorptive properties may indicate a potential for both bioaccumulation and biomagnification. For those chemicals where the log K_{ow} cannot be measured properly, a high adsorptive capacity (log K_{oc} > 3) can be additional evidence of bioaccumulation potential.

Degradation

Both biotic and abiotic degradation may lead to relatively low concentrations of a substance in the aquatic environment and thus to low concentrations in aquatic organisms. In addition, readily biodegradable substances are likely to be rapidly metabolised in organisms. However, the uptake rate may still be greater than the rate of the degradation processes, leading to high BCF values even for readily biodegradable substances. Therefore, ready biodegradability does not preclude a bioaccumulation potential. If persistent metabolites are formed in substantial amounts, the bioaccumulation potential of these substances should also be assessed.

Metabolism

If it appears that the BCF of a substance lies significantly below the estimate from the (Q)SAR (e.g. more than one log unit), this is a strong indication for metabolism of the compound. Information on possible degradation products can be obtained by using expert systems (e.g. Catalogic, which can predict biodegradation pathways and metabolites) or by conducting *in vitro* metabolism (e.g. fish microsomes and S9 fraction) or *in vitro* transport tests (e.g. Caco-2, perfused gill, fish intestinal preparations). These test methods may become an important part of future test strategies, but their applicability is currently limited due to the lack of standardised protocols, limited validation based on small data sets. Initiatives such as the ILSI HESI Bioaccumulation project (ILSI HESI website) are working on developing tools needed for proper assessment of bioaccumulation potential.

BCF (Q)SAR models

The most common and most simple (Q)SAR models for estimating BCF are based on correlations between BCF and chemical hydrophobicity (as modelled by log K_{ow}). The mechanistic basis for this relationship is the analogy of the partitioning process between lipid-rich tissues and water to that between n-octanol and water (whereby n-octanol acts as a lipid surrogate). In this model, uptake is considered to be a result of passive diffusion through gill membranes. These early, conservative models have been vastly improved via the incorporation of metabolism of the test substance into the models (Arnot et al, 2008a; 2008b). Chemicals that are ionised at environmental pH values are generally outside the applicability domain of current bioaccumulation (Q)SAR models. For those (Q)SAR models that provide a list of compounds similar to the target chemical (e.g. CAESAR BCF model), one should always check the structure of the similar compounds to determine if the prediction can be deemed reliable (e.g. using similarity indices). (Q)SAR models for predicting fish BCFs have been extensively reviewed in the literature (e.g. Boethling and Mackay, 2000; Dearden, 2004; Pavan et al, 2006) and also in the ECHA technical guidance Chapter R.7c (ECHA REACH TGD), and are not discussed further in this report.

There are several factors that can affect bioaccumulation in addition to metabolism, and some of these can be considered on an individual compound/class basis. These include active transport phenomena, accumulation potential of metabolites, affinity to specific tissue components, e.g. protein storage sites, uptake and depuration kinetics. Efforts are continually underway to incorporate improvements in predictability into all available models (e.g. newest version of BCFBAF which incorporates metabolism).

Molecular mass and size

The PBT Working Group, established under the (former) ECB Technical Committee on New and Existing Substances (TC NES) has recommended that the following indicators either alone or in combination indicate that chemicals may not bio-concentrate to a level of concern, recognising the uncertainties in the interpretation of experimental results:

- a molecular weight of >1100 g/mol suggests a chemical is not vB (i.e. BCF < 5000);
- a maximum molecular length of 43 Å indicates no uptake and indicates a chemical is not B and not vB;
- a maximum cross-sectional diameter of 17.4 Å indicates a chemical is not vB (i.e. BCF <5 000);
- a maximum cross-sectional diameter of 17.4 Å plus a molecular weight of 700 - 1100 g/mol suggests a chemical is not B (i.e. BCF < 2000);
- a measured octanol solubility (mg/l) < 0.002 * MW (assuming baseline toxicity) indicates a chemical is not B.

Test data

For substances with log K_{ow} values between 1.5 and approximately 5, the OECD 305 (2012d) guideline (equivalent to ASTM E1022-94 and OPPTS 850.1730) is the preferred test to conduct. A dietary method has been incorporated into the latest version of OECD TG 305 to account for poorly water soluble substances for which aqueous exposure is technically difficult. Before carrying out read-across, it is thus important to check whether the appropriate test guideline has been followed and if the test if valid.

WoE for bioaccumulation

Building a weight of evidence case for bioaccumulation involves two basic stages: data gathering and decision stage. By taking into consideration the factors described in the previous section, the following steps can be followed as discussed in Nichols et al (2009). See also Appendix A for a workflow outlying the steps.

Data gathering

i. Collect information regarding factors that may contribute to or mitigate the potential for bioaccumulation, e.g. physicochemical properties, chemical structure info, fate information (hydrolysis rate – not inherently accounted for by bioaccumulation models) and biodegradation potential.
ii. Run all available bioaccumulation models relevant for chemical prioritisation or assessment. The models should incorporate ADME information from *in vitro* and *in vivo* data sources when available.

iii. Verify output for reliability (domain of applicability).
iv. Examine results in the context of the physicochemical properties, chemical structure and fate info to identify compounds that may be difficult to model.
v. Results deemed to be reliable can then be combined using qualitative or quantitative weighting schemes in the decision stage.

Decision stage

In most cases, a qualitative approach is used to weigh decisions based on model output given the uncertainties inherent in this information. A simple approach would involve taking the highest model prediction as a final value to use for prioritisation or assessment. This conservative approach results in fewer false negatives but potentially many false positives. False positives and false negatives can be minimised by seeking consensus among the models. When predictions from the models are reliable but are not in agreement, taking a decision may involve weighing results based on regulatory data requirements and would be context dependent.

Quantitative weighting methods employ statistical or semi-quantitative methods to rank the bioaccumulation potential of one chemical over another (e.g. partial order ranking, multidimensional statistics). These methods are best suited to ranking large inventories of chemicals for prioritisation purposes rather than single chemical assessment.

6.5.5 Acute mammalian toxicity

Oral route

Acute mammalian toxicity comprises many different modes of action. Encoding these modes of action to chemical features has largely been hampered by availability of relevant acute toxicity information. Whilst statistical models such as TOPKAT and MCASE predict overall LD_{50} values in rats with differing degrees of success, most efforts have been limited to local (Q)SAR for specific chemical classes (Tsakovska et al, 2008). In such cases, the close structural similarity in terms of common functional groups, common Cramer structural classes and a similarity in key physicochemical profiles in addition to a commonality in steric, hydrophobic and electronic features may provide sufficient evidence to justify a read-across. *In vitro* cytotoxicity testing that has been shown to be predictive of acute oral toxicity in rodents might be an additional complement to substantiate the further read-across. This type of strategy would likely suffice in the case of a category based on strong structural similarity such as common functional group. If the category was based on chemical or metabolic transformation in particular, then empirical data to demonstrate that the transformation occurred (*in vitro* metabolism or hydrolysis) and resulted in the formation of a metabolite/degradate would provide convincing evidence that information on the degradate was most relevant to use. For example, an anhydride or acid chloride is likely to hydrolyse to its corresponding acid; providing a hydrolysis study to demonstrate evidence of hydrolysis would substantiate a read-across to the acid. An ester that was subject to metabolism and would be converted to its corresponding alcohol would benefit from data from at least an *in vitro* metabolism study to substantiate the rationale that the alcohol was the most relevant substance to evaluate.

Case study metals: Read-across for acute oral toxicity

Reliable data for the assessment of acute oral toxicity of $BaCO_3$ are not available. However, the toxicity of barium substances such as barium carbonate can reasonably be assumed to be determined by the availability of barium ions in solution. This was investigated for barium carbonate experimentally in a test for comparative bioaccessibility with barium carbonate and barium chloride in artificial gastric juice (HCl, pH=1.5): an excess of each test item was added to freshly prepared HCl solutions (pH=1.5) to obtain saturation. It could be shown that the solubility of barium carbonate in acidic media at 37°C is 3.7 g/l, whereas 510.4 g/l of barium chloride could be dissolved under equal conditions. In consequence, the solubility of barium carbonate under these conditions is more than two orders of magnitude less than that of barium chloride. Therefore, it was decided to waive acute oral toxicity testing of barium carbonate and to read across from barium chloride. The LD_{50} of barium carbonate has been calculated stoichiometrically from the available LD_{50} of barium chloride. However, it should be kept in mind that read-across from barium chloride to barium carbonate is inherently very conservative.

Inhalation route

There are a number of factors to consider when evaluating acute inhalation toxicity. For volatile substances, key factors include physicochemical parameters such as vapour pressure, water solubility in addition to chemical reactivity (Veith et al, 2009). For non-volatiles, particle size is critical. Solid aerosol particles can be generated as dusts, fumes, smoke or granule. Liquid aerosols can be generated as mists or fogs by spraying, nebulisation or by pouring. If the particles that are generated as a result are small (less than 100 µm), they may be respirable and hence pose a particular hazard. Metabolism may also be a factor; the lung contains most of the same enzymes that are encountered in the liver. Some P450 enzymes are more abundant in the respiratory tract.

For volatiles, if the source and target are presumed to be general narcotics, and possessed no electrophilic functionality as such, one could expect the inhalation toxicity to be reasonably comparable based on their similar physicochemical characteristics. Whilst fish and mammalian inhalation baseline toxicity are not directly comparable because their external media are different, their blood thermodynamic activity for LC_{50} (narcosis) is the same in both fish and mammal. At steady-state, the activity in air/water equals the activity in blood by definition: $\alpha = C \times \gamma$ where α: activity; C: concentration; γ: activity coefficient.

If activity for narcosis in fish and rat were equal, a plot of LC_{50} versus solubility in exposure medium should be the same. The study by Veith et al (2009) has shown that this is indeed the case for baseline general narcotics.

Substantiating a read-across for inhalation effects of volatiles could borrow much from other endpoints where narcosis and electrophilic reaction schemes play a role. For example, a substance believed to behave as a general narcotic for inhalation might benefit from a structural assessment based on profiling tools within the OECD (Q)SAR Toolbox to demonstrate a general narcosis mode of action was indeed relevant. Here, Verhaar et al (1992) or the OASIS MOA for aquatic toxicity could prove useful. If the estimates from a (Q)SAR evaluation based on a general neutral MOA were consistent with experimental data, then the MOA could be substantiated. Additional evidence of a neutral organic MOA might arise from the absence of effects in sensitisation and mutagenicity assays such as Ames which are reliant on a covalent reaction

mechanism. If narcosis was not the mode of action but reactivity did indeed play a role, use of an *in chemico* assay might provide supporting information that the target substances were giving rise to unspecific reactivity via a covalent reaction mechanism. In this case, supporting information from a relevant (Q)SAR for aquatic acute toxicity that was in good agreement with experimental data coupled with sensitisation and mutagenicity data should substantiate an covalent reaction scheme and suggest that inhalation effects are in excess of those predicted by vapour pressure alone.

For non-volatiles, consistency in potency across other routes of exposure, dermal and oral, would also help to substantiate a read-across.

6.5.6 Irritation

Skin and eye irritation are endpoints where there has been significant progress in developing *in vitro* alternatives which minimises the need for any *in vivo* testing. Some efforts have been made in estimating irritation potential quantitatively through chemical class-specific (Q)SAR where the chemical-determining features have focused on hydrophobicity (often modelled by log K_{ow}), reactivity (dipole or pKa) and size (molecular weight MW or volume) (Gallegos-Saliner et al, 2008b).

Skin irritation

Validated *in vitro* tests exist for both skin corrosion and skin irritation. The MOA for skin corrosion/irritation have been discussed by Walker et al (2005). The presence of skin corrosion/irritation SARs have been established by Hulzebos et al (2005) The rules for the presence of skin irritation have been evaluated by Gallegos-Saliner et al (2008b). Using analogue approaches for skin irritation is mainly feasible for inferring the presence of skin corrosion or irritation. In addition to the available structural alerts, the following parameters can be included to further underpin the reasoning:

- Acidity (estimated by pKa)
- Basicity/alkalinity (estimated by pKa using SPARC or ACDLabs)
- Chemical burns (isocyanates, mustards) [for eye irritation in particular]
- Interaction with proteins (metal salt deposition, quinones, etc.) [for eye irritation in particular] could be identified by reaction rules encoding for covalent reaction binding (OECD (Q)SAR Toolbox, Toxtree or Derek Nexus could be utilised in this regard)
- Mechanical abrasions
- Solvent effects
- Surfactancy

Under REACH, specific adaptations exist to waive the need for irritation testing specifically using pH; chemicals with pH < 2 or > 11.5 can be classified as corrosive without any further testing.

In the case of an analogue approach for predicting the absence of skin corrosion and/or skin irritation, a read-across may need further substantiation using expert judgment on the absence of reactivity or using SARs. The physicochemical rules for the absence of skin corrosion/irritation have been established by Gerner

et al (2005). The rules for the absence of skin corrosion/irritation have additionally been evaluated by Rorije and Hulzebos (2005).

Both these rulebases have been encoded in Toxtree and the OECD (Q)SAR Toolbox. Additional SARs are also available within the Derek Nexus system.

Eye irritation

For eye irritation, progress in developing *in vitro* alternatives has been accomplished. A validated eye corrosion test is available. However, a validated test for the assessment of absence and presence for eye irritation is not yet available.

Using analogue approaches for eye corrosion/irritation is mainly feasible for predicting the presence of eye corrosion/irritation. Many of the same parameters as for skin irritation may be included to further underpin the reasoning:

- Acidity based on pH (and/or estimated by pKa);
- Basicity/alkalinity on pH (and/or estimated by pKa);
- Chemical burns (isocyanates, mustards) [for eye irritation in particular];
- Interaction with proteins (metal salt deposition, quinones, etc.) [for eye irritation in particular] could be identified by reaction rules encoding for covalent reaction binding;
- Mechanical abrasions;
- Solvent effects;
- Surfactancy.

For absence of eye irritation using an analogue approach, an estimate needs to be substantiated with additional information from non-validated *in vitro* tests (the ICE test) and/or (Q)SAR which can predict the absence of effects for a specific group of chemicals (Worth and Cronin, 2003). Gerner et al (2005) has presented physicochemical rules for the absence of eye irritation and structural alerts for the presence of eye corrosion and eye irritation which were validated by Tsakovska et al (2005; 2007).

6.5.7 Skin sensitisation

A skin sensitiser is an agent that is able to cause an allergic response in susceptible individuals. The key steps required for a chemical to induce skin sensitisation are gaining access to the viable epidermis, protein binding, metabolic activation (if required), internalisation and processing by Langerhans cells (LC), transport of antigen by LC to draining lymph nodes, and presentation to and recognition by T lymphocytes. In this process, the key factor is the potential to bind to proteins in such a way that this protein can be transported. Therefore, (Q)SAR and especially *in vitro* methods mostly focus on this aspect (Mehling et al, 2012). This step is assumed to be the rate determining step or MIE. Though dermal availability is a key parameter in the process for a skin sensitisation reaction, a cut off for the absence of dermal availability has not been established (Zinke et al, 2002).

Skin sensitisation is a local and systemic endpoint, because the reaction occurs at the site of entry. It is a systemic endpoint because first of all systemic immunological reaction follows upon a first exposure. At later exposures, a reaction is seen on the skin where the exposure occurs which is not necessarily at the same spot where the first exposure was.

MOA

Protein binding is assumed to be covalent whereby the chemical behaves as an electrophile and the protein as a nucleophile. The hypothesis was first articulated in 1936 by Landsteiner and Jacobs (1936) followed up by others including Godfrey and Baer (1971) and Dupuis and Benezra (1982). For effective sensitisation, a chemical must either be inherently protein reactive or be converted (chemically or metabolically) to a protein-reactive species. Chemicals that are unable to associate effectively with proteins fail to stimulate an immune response. Efforts to predict skin sensitisers have hence been focused on identifying the electrophilic features in chemicals and relating these back to skin sensitisation potential.

There are numerous reviews articulating the availability of (Q)SAR for skin sensitisation that focus on the identification of electrophilic features (see Patlewicz and Worth [2008] for one such review). For potential sensitisers, so-called mechanistic classes have been developed, which contain a variety of structures. Aptula et al (2005) evaluated a data set of 41 LLNA (local lymph node assay)EC3 values for a diverse range of compounds and re-classified them into reaction mechanistic applicability domains, i.e. grouping according to the reaction mechanisms whereby the compounds could react with nucleophiles. Applying these mechanistic organic chemistry principles, revealed clear trends, within each domain, where sensitisation potential increased with increasing reactivity.

The major reaction mechanistic applicability domains identified were: Michael-type addition, S_N2, S_NAr, acylation, and Schiff-base. Included in the Michael-type domain were pro-Michael acceptors, these being compounds which are not themselves Michael-reactive but are easily converted (e.g. by *in vitro* or *in vivo* oxidation to Michael acceptors). There was also an 'unreactive' domain; compounds in this domain are expected to be non-sensitisers. Whilst these are not the only mechanistic applicability domains, they did provide a foundation to underpin skin sensitisation with sound reaction chemistry principles which were documented more fully in Aptula and Roberts (2006) as a set of rules for characterising each domain. The rules were subsequently used to evaluate other datasets as evidenced in Roberts et al (2007), amongst other examples. An addition might be to include a domain of S_N1 based on some examples identified where S_N1 reactions appear to offer a plausible explanation for behaviour. Examples include tertiary allylic peroxides, for which protein binding by free radical reactions has also been suggested (Karlberg et al, 2008; and references therein).

These mechanistic classes and similar are included in several expert systems such as TIMES and Derek Nexus. The original Aptula and Roberts (2006) mechanistic classes were encoded as SMARTs alerts by Enoch et al (2008) into Toxtree. These have been extended and evaluated to formulate the current protein binding alerts that exist as profilers within the OECD (Q)SAR Toolbox (Enoch et al, 2010a). Though these mechanistic classes may contain a variety of chemical structures, for the purpose of read-across within analogue and category approaches, under REACH or other regulatory programmes in addition to commonality of reaction domain, some tenet of structural similarity is preferred.

Tools

Several tools can be used to help characterise the analogue and category approach further: databases, (Q)SAR, *in vitro* testing, and similarities in (eco)toxicological profiles other than sensitisation.

Databases

It is likely that using an analogue approach to infer the presence of skin sensitisation requires less information than that needed for the absence of skin sensitisation. Databases on skin sensitisation such as those implemented into the different expert systems or within the OECD (Q)SAR Toolbox may prove helpful to identify additional analogues to substantiate a read-across. In 2010, a second compilation of LLNA data has been published by Kern et al (2010) and contains data for over 300 substances.

(Q)SAR

Using available (Q)SAR to show that target and the source analogues have similar structural and toxicological features is also helpful.

Toxtree and the OECD (Q)SAR Toolbox which encode a number of skin sensitisation alerts as described above provide qualitative indication of similarity in reaction domain. Derek Nexus which predicts skin sensitisation based on a number of structural alerts can be useful to show that both target and source analogue(s) share similar structural alerts by virtue of the same confidence in the prediction of skin sensitisation. TIMES predicts both the presence and absence of skin sensitisation as well as semi-qualitative potency score. A comparable prediction between the target and the source should be seen. The following three statistical systems may be helpful to demonstrate related analogues. Caesar, TOPKAT and MCASE all predict activity and non-activity for skin sensitisation. The selection of similar chemicals should be the same for both target and analogue substances.

In chemico-in vitro

To substantiate the read-across further, a measurement of reactivity would provide convincing evidence of similarity in reactivity and thus sensitising potency. Examples of *in chemico* and *in vitro* assays are described by Schwöbel et al (2011) and Mehling et al (2012). A number of assays have been undergoing ECVAM validation; these may also provide corroborating evidence to support the read-across. The most promising tests as presented in the ECVAM report of 2010 are the Peptide Reactivity Assay (DPRA), the human Cell Line Activation Test (h-CLAT) and the Myeloid U939 Skin Sensitisation Test (MUSST) (see http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/animal_testing/at_ecvam_2008-2009_en.pdf).

It is worth noting that these assays will be reconsidered in light of AOPs as part of the OECD work programme since whilst the DPRA measures reactivity per se, the remaining two measure other key events in the progression of skin sensitisation induction.

Consistency in hazard profile based on common MIEs

Another consideration to demonstrate similarity in behaviour could be by virtue of the common MIE, i.e. electrophilicity with other surrogate endpoints such as genotoxicity or aquatic toxicity. Whilst there are some differences in metabolism for genotoxicity and sensitisation, there is a large overlap in the structural alerts that trigger both endpoints. An extensive evaluation analysing the alerts and rationalising them in light of available sensitisation and genotoxicity was undertaken by Mekenyan et al (2010) and evaluated in light of a data set in Patlewicz et al (2010). The Cramer classes are also potential indicators for toxicological reactivity and may give a preliminary indication whether the substance is (non) reactive.

MOA profilers for ecotoxicity such as the Verhaar et al (1992) scheme might also provide complementary information.

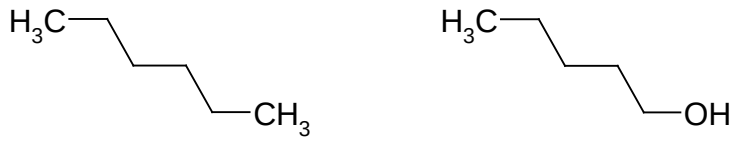
Overall, in the evaluation of a read-across approach for the skin sensitisation endpoint, there are many strategies to exploit that are helpful to build a WoE approach. In addition to the available expert systems for the prediction of skin sensitisation, an assessment can be made of likely reaction mechanistic domains making use of the available profilers such as those contained within the OECD (Q)SAR Toolbox and substantiating the reactivity by virtue of *in chemico* or *in vitro* reactivity assays as well as corroborating information derived from genotoxicity *in vitro* assays or ecotoxicity assays.

Whilst reactivity is a key factor in sensitisation potential, dermal bioavailability between the target and source analogues should be considered as part of the comparison as well as in the interpretation of in available *in vivo* data. Physicochemical properties such as molecular weight, water solubility, log K_{ow} and vapour pressure are important to consider. For the skin sensitisation endpoint, these toxicokinetic properties cannot be used to exclude skin sensitisation (e.g. in contrast to skin irritation properties), because some high molecular weight and high log K_{ow} chemicals may still cause skin sensitisation, e.g. long-chain amines (Zinke et al, 2002), and some substances may give rise to misleading results in one of the other assays due to volatility (Poryazova et al, 2012).

Note, the *in vitro*/*in chemico* experimental strategies work for direct acting sensitisers only. Clearly a number of chemicals show skin sensitising properties because of their metabolites or due to chemical oxidation. For example, cinnamic alcohol can be converted to cinnamic aldehyde which is a sensitiser (Gerberick et al, 2008). Phenylene diamine is also a sensitiser by virtue of oxidisation to an imine form (Gerberick et al, 2008; Roberts et al, 2007). Strategies to assess potential breakdown products for their sensitising potency have been incorporated into tools such as TIMES. In addition to its skin metabolism simulator (which is also implemented in the OECD (Q)SAR Toolbox), work is underway in implement an autoxidation simulator. A version of this will be made available in the Toolbox v3 currently due for release in October 2012.

Whilst it is perhaps more difficult to prove that a chemical is not a sensitiser, because all skin sensitisation reaction pathways would need to be known before such a statement can be made, there are some practical strategies to consider in the identification of non-sensitisers. Generally, chemicals without any activating groups, e.g. alkanes, benzylic rings and alcohols, are not sensitisers. In ecotoxicity they are often referred to as neutral organics. Hexane and propanol-alcohol shown in Figure 4 are examples of this group.

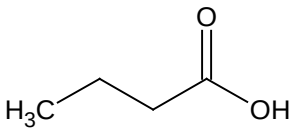
Figure 4: Examples of non-sensitising chemicals



A chemical may be non-reactive due to the lack of any activating groups, e.g. an ester without any adjacent groups to activate this.

Non-sensitising chemicals may also be too reactive, for example strong acids and bases or other strong reactants which are too cytotoxic to form a covalent bond. An example is butanoic acid shown in Figure 5.

Figure 5: Butanoic acid as an example of a chemical that is too reactive to form a covalent bond



6.5.8 Respiratory sensitisation

Respiratory sensitisation can be assessed using human data such as indicated in the REACH guidance. If no human data are available, respiratory sensitisation can be assessed using the integrated evaluation strategy:

- If the chemical is not a skin sensitiser, then it is unlikely to be a respiratory sensitiser.
- If the chemical is a (weak) skin sensitiser, then it needs to be assessed whether the chemical is a (di-)isocyanate. In addition, the structural relation with other chemicals causing respiratory sensitisation needs to be addressed (e.g. iso-thiocyanates, amines, anhydrides, acrylates, diazonium salt and reactive dyes, metals).

When the chemical is a non-skin sensitiser or it is a skin sensitiser but the above criteria are not fulfilled, it can be concluded that the chemical is not a respiratory sensitiser. Work is underway to explore *in chemico* reactivity for respiratory sensitisation purposes. Examples have been published by Enoch et al (2010b) and Lalko et al (2011). This type of information which is generated for skin sensitisation may be equally complementary and corroborating for the purposes of respiratory sensitisation.

6.5.9 Mutagenicity

As discussed already, the rate determining step of skin sensitisation induction is thought to be the formation of a covalent bond between the electrophilic chemical and the skin protein nucleophile. However, covalent bond formation as a rate determining step is not unique to skin sensitisation. Schultz et al (2006) described a conceptual framework for predicting the toxicity of reactive chemicals where plausible molecular initiating

events (MIEs) were based on covalent reactions with nucleophiles in proteins or DNA, and would ultimately lead to a variety of different adverse outcomes such as aquatic fish toxicity, mutagenicity, hepatocyte cytoxicity or respiratory toxicity. Electrophilicity is well known to be an important factor in driving mutagenicity and carcinogenicity (Miller and Miller, 1977). They found that it was possible to rationalise the activity of a large majority of animal carcinogens at the time on the basis of their electrophilic potential. The activity of chemicals as mutagens to *Salmonella typhimurium* almost always seems plausible within the context of the Millers' hypothesis. For predictive purposes, electrophilic features are readily encoded into structural alerts (SA). Seminal efforts include SA for carcinogenicity by John Ashby (1985), who subsequently extended his list with additional SA (Ashby and Tennant, 1991). Bailey et al (2005) compiled a set of 33 SA for regulatory use within the US Food and Drug Administration (FDA), which was predominantly based on the Ashby alerts. Kazius et al (2005) evaluated a mutagenicity database comprising 4337 mutagens and non-mutagens taken from the Toxnet database (http:/toxnet.nlm.nih.gov/) and derived 29 SA for mutagenicity with associated detoxification fragments. Some of these alerts exist in software platforms to enable routine use, e.g. 17 SA for mutagenicity are implemented into the OASIS TIMES software (Mekenyan et al, 2004). Benigni and Bossa (2008) combined the published information from Ashby, Bailey et al, Kazius et al with additional information from the OncoLogic™ (US EPA) software (http://www.epa.gov /oppt/sf/pubs/oncologic.htm) to arrive at a list of 33 SA for carcinogens and mutagens that were implemented in Toxtree as well as the OECD Toolbox. Other efforts include SAs that have been encoded into the OECD Toolbox as DNA Binding Profilers (Enoch and Cronin, 2010) to facilitate category formation. These authors have since proposed 26 new alerts for genotoxicity and non genotoxicity (Enoch and Cronin, 2012). Current quantitative strategies include (Q)SARs and expert systems. Two types of (Q)SAR models, local and global, exist to estimate the mutagenic potential of chemicals. Local (Q)SARs provide estimated results for closely related (congeneric) chemical structures. Examples include that by Chung et al (1997), more are reviewed in Benigni et al (2007).

Global (Q)SARs aim to provide mutagenicity estimations for a diverse (non-congeneric) set of chemicals. Such (Q)SARs may be additionally encoded into expert systems. For example, TOPKAT empirically makes predictions for a range of different endpoints including Ames mutagenicity (Serafimova et al, 2010). Other expert systems such as TIMES attempt to provide clear mechanistic meaning through the use of SA which address the reactivity towards DNA and/or proteins. TIMES also includes 3D QSARs to underpin some of the available SA (Serafimova et al, 2007). All the aforementioned (Q)SARs have typically been derived on Ames (*Salmonella* mutagenicity data). TIMES includes a platform for *in vitro* chromosomal aberration data in addition to that for Ames (Mekenyan et al, 2007). There is a paucity of models for *in vivo* genotoxicity but as highlighted in the survey by Benigni et al, there is only one publically available model for *in vivo* micronucleus (Benigni et al, 2010). The scarcity of such models may be due in part to experimental data being less readily available, but also due to the complexity of how to rationalise and interpret the outputs from the different test systems. Efforts have been made in developing expert systems for *in vivo* genotoxicity, further refinements to TIMES now includes models for *in vivo* liver genotoxicity and *in vivo* micronucleus prediction (Mekenyan et al, 2012). Substantial efforts were made in trying to rationalise the differences between *in vitro* and *in vivo* results on the basis of metabolism.

Mutagenicity information is effectively a useful surrogate for chemical reactivity. Based on the analysis performed in Mekenyan et al (2010), a positive result in either Ames and/or *in vitro* CA provides compelling evidence that a substance of interest is a likely sensitiser and this information should be considered as part

of the weight of evidence approach for hazard identification. Conversely then information from a sensitisation study can provide practical insights into the expected outcomes in these mutagenicity assays and therefore could be useful in a read-across justification.

Overall, electrophilicity is a key feature as encoded in the available SA. There are well established alerts to help rationalise outcomes in Ames and a handful of models that can aid in the evaluation of other genotoxicity assays. Exploiting commonality in MIEs and the associated information from studies such as sensitisation can also be helpful.

6.5.10 Repeated-dose toxicity

Repeated-dose toxicity is a complex endpoint and one where a paucity of mostly local (Q)SAR has been attempted. Derek Nexus presents structural alerts for specific target organ toxicity. TOPKAT purports to predict a LOAEL. As far as non-testing approaches go, the following strategies could be undertaken in support of a read-across. The Cramer structural classes as encoded in Toxtree and the OECD Toolbox would serve to indicate the likely concern level of the target and source analogue(s). For a read-across, the expectation is that all analogues would flag the same Cramer structural class. TOPKAT may also be useful to demonstrate consistency in predictions by giving rise to a comparable set of similar analogues from its training set. The Toolbox is pursuing an AOP approach for repeated-dose toxicity by encoding a new repeated-dose profiler based on data received from Japan. The profiler and the database will be integrated in Toolbox v3 due for release in October 2012. In the interim, the HESS database and the corresponding profiler can be used as stand-alone tools:

Link to HESS: http://www.safe.nite.go.jp/kasinn/qsar/qsar_files/NITE_HESS_May_2012.zip
Link to HESS DB: http://www.safe.nite.go.jp/kasinn/qsar/qsar_files/NITE_HessDB_May_2012.zip

In addition to structural alert information as reflected in the above tools, the oral, dermal and inhalation bioavailability and the similarity between the target and the source chemicals need to be evaluated. This can be addressed, to an extent, by physicochemical information using parameters such as molecular weight, water solubility, log K_{ow} and vapour pressure especially in cases where no experimental toxicokinetic data are available. In addition, the effects seen in other *in vivo* testing can be used to demonstrate whether the substance is even systemically available. This can be achieved through assessing the effects observed in the acute tests or other repeated-dose toxicity testing (such as the 28-day test). If the substance is an irritant or a sensitiser, some dermal availability may also be expected.

A discussion on the target and source chemical's reactivity to target organs is needed based on available experimental data. *In vitro* assay information on cytotoxicity may also be useful. In the ECETOC TR109 report (2010), several methods are described on how high throughput screening methods, toxicogenomics and metabolomics can be helpful surrogates to substantiate read-across approaches. Comparable information can be found in Voutchkova et al (2010).

It is worth noting that the activity of certain classes of organic chemicals may differ based on the substitution position, i.e. where the functional groups are attached to a ring system. Ortho-, meta- and para-substituents

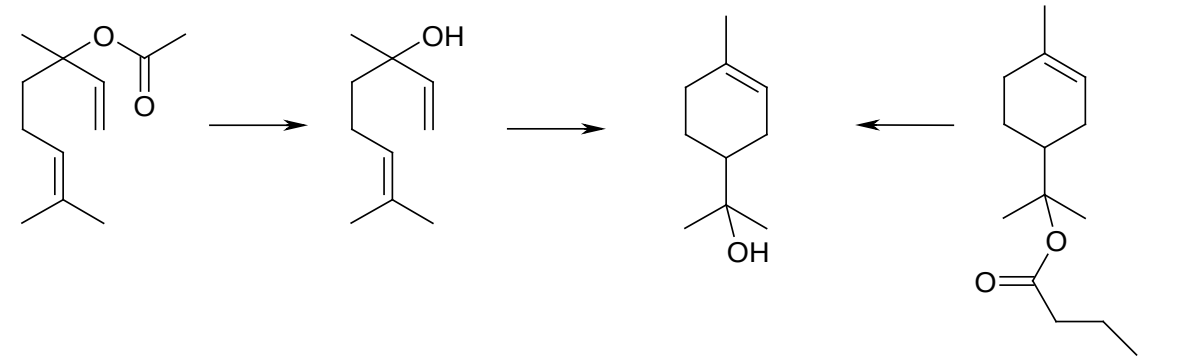
on cyclic and benzylic rings are expected to have different stability and also different toxicological outcomes. Though for the xylene category this was not shown: http://webnet.oecd.org/hpv/UI/handler.axd ?id=7f6b4807-5217-4626-b47c-e139327a412b. DeVito (1996) has presented a number of interesting cases on these differences. Double bonds and their position on the structure can be of minor or major influence. Double bonds at the end of a carbon chain as from olefins (e.g. C-C-C=C) may result in e.g. epoxide formation. Allylic alcohols or their related ethers or esters, that contain at least one hydrogen on the alcoholic carbon (C=C-C-OH) are also more reactive compared to a sole double bond within a chain, because of the formation of α β-unsaturated carbonyl metabolite (e.g. DeVito, 1996 and updated by Voutchkova et al, 2010).

One also needs to evaluate whether isomers, trans and cis, R and S may have different toxicological outcomes due to the fact that these isomers may bind differently to receptors and thus influence receptor-mediated pathways. The function of specific isomers has been shown in drugs, agrochemicals and fragrances. The toxicological importance is less known except for one important example being thalidomide (softenon). For the different isomers of the menthols, no difference in toxicity was observed (http://www.inchem.org/documents/sids/sids/MENTHOLS.pdf)

When the analogue or category approach is based on the formation of similar metabolites, the formation of metabolites needs to be explained in detail. Probably the most common metabolic category is based on esters which metabolise to corresponding alcohols and acids. For the local endpoints (e.g. skin and eye irritation and fore stomach irritation) both the parent substance and the metabolites need to be discussed. For esters it can be assumed that partly by hydrolysis but even more so by esterases, which are abundantly available in the gastrointestinal tract, the lungs and the skin, the parent chemical metabolises extensively. This esterification pathway can be saturated and therefore experimental testing with high doses may not always reflect realistic exposures.

A number of examples are available where esters are used as a metabolic category. In the OECD HPVC SIDS programme, the categories formates and phosphates esters and several others have been presented. Belsito et al have presented the cyclic acetates and the salicylates (Belsito et al, 2007; 2011). Wu et al (2010) have presented the category of the terpinylacetate and terpineol (see Figure 6). They took even a bigger step and showed a category of linalylacetate, linalool, terpineol alpha and terpineol acetate. Linalyl acetate and linalool are the non-cyclic members of the category and the terpinyls being the cyclic members.

Figure 6: The metabolic category presented by Wu et al (2010)



Linaylacetate Linalool alpha-Terpineol alpha-Terpinylpropionate

A similarity in the overall toxicological profile, i.e. across a number of mammalian endpoints, may help support the justification of the read-across of repeated-dose toxicity. Skin and eye irritation can denote reactivity and potentially some local effects such as irritation in the forestomach. A similarity in skin sensitisation and genotoxicity profile will also provide an indication of reactivity which can be helpful information to support the repeated-dose read-across.

General considerations when performing read-across for repeated-dose toxicity

As with all other endpoints, demonstrating a consistent toxicological profile across the group, or identifying trends and the properties that drive any trends are key in building a successful read-across justification for repeated-dose toxicity.

However, when using read-across as part of a category or analogue approach to address repeated-dose toxicity endpoints, one must recognise that due to the greater potential complexity associated with these endpoints, and their importance in risk assessment, the justification for read-across needs to be more comprehensive than for endpoints such as irritation, sensitisation and *in vitro* genotoxicity where one can rely to a far greater extent on the predictions from SARs to support the category. This does not mean that in order for a read-across argument to be considered robust, one must generate substantial repeated-dose toxicity data on every member. If that was the case then the use of categories would not have the desired function of allowing hazard characterisation of multiple substances without resorting to additional testing.

While keeping in mind the desire to minimise additional testing where possible, generating sub-acute repeated-dose toxicity data on each category member is the most comprehensive approach to building a category supporting read-across for repeated-dose toxicity. In doing so, an assessment of toxicokinetics can be built in (refer to Chapter 7), providing further support for grouping substances together. In taking this approach, a significant investment is made into grouping the substances. However, this is offset by the benefits of using read-across to address the higher tier repeated studies, the 90-day and chronic studies. Although this is essentially an 'idealistic' approach since every member has some data, there would still be uncertainty involved in reading across for the longer term studies since effects in these longer duration and more comprehensive studies may not be identified or assessed in sub-acute studies. Therefore, it is

important to consider whether longer term studies are needed on multiple members of the category to reduce the uncertainty that hazards of the category members could be missed.

Non-animal tests such as *in vitro* or (Q)SAR tools can be useful in supporting a read-across approach for repeated-dose toxicity. At this time, there are no suitable *in vitro* methods that can replace repeated-dose testing, nor is this likely to happen within the near future. However, *in vitro* tests addressing mode of action or potency can still be highly valuable when forming a read-across justification.

As with *in vitro* assays, there are no (Q)SAR tools that alone can predict the outcome of repeated-dose toxicity studies. However, (Q)SAR tools and databases can be used to strengthen the read-across for repeated-dose toxicity (OECD (Q)SAR toolbox, Repdose database, etc.) in situations where there are some repeated-dose toxicity data on the category members or analogues. Although these tools cannot reliably predict a 'no observed effect level', they can be used to demonstrate that structurally similar substances show a similar pattern of repeated-dose toxicity, with consistent target organs, potencies etc.

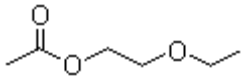
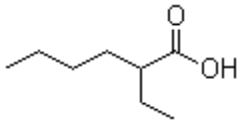
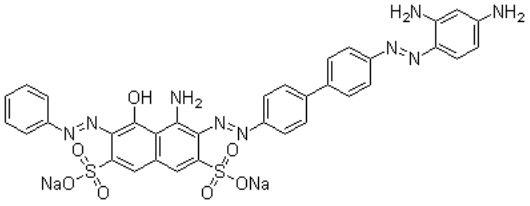
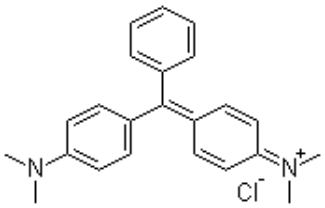
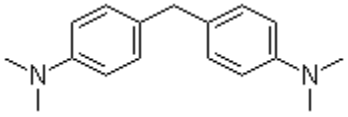
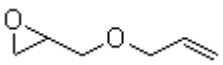
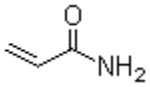
Where there are limited repeated-dose toxicity data available across category members, in addition to (Q)SAR tools, other data such as acute toxicity, irritation, sensitising potential and genotoxicity can be used to demonstrate similarity in reactivity or cytotoxicity that can help underpin the read-across for repeated-dose toxicity.

6.5.11 Reproductive and developmental toxicity

Only a few (Q)SAR methods are available to assess reproductive toxicity. An overview is presented by Lo Piparo and Worth (2010). The OECD (Q)SAR Toolbox presents a profiler for oestrogen receptor binding. Caesar/Vega presents a tool to predict developmental toxicity. The data for this tool have been presented by Hewitt et al (2010).

A limited number of SARs are available that are known to express reproductive and developmental toxicity. A summarised overview from Maslankiewicz et al (2005) is presented in Figure 7, which includes the US-EPA-TSCA potential reproductive and developmental toxic classes. The number in brackets cites the number of chemicals found in the 29th ATP (according to Directive 67/548/EEC, 1967).

Table 4: Reproductive and developmental SARs from Derek for Windows (2005) and US-EPA-TSCA (from Maslankiewicz et al, 2005)

Structural alerts present in DEREKfW 8.0	Chemical Categories in the TSCA List
Monothioglycol or glycol monoalkyl ether, alkoxy-or alkylthio-carboxylic acid or precursor (9) e.g. CAS no. 111-15-9 	Ethylene Glycolethers (10) e.g. CAS no. 111-15-9
Short-chain carboxylic acids or precursor e.g. CAS no. 149-57-5 	Anhydrides Carboxylic acid
Benzidine based bisazocompounds (3) e.g. CAS no. 1937-37-7 (C.I. Direct Black) 	Triarylmethane pigment dyes with non-solubilising groups (1) e.g. CAS no. 569-64-2 
	Dianilines (1) e.g CAS no. 101-61-1 
	Epoxides (5) e.g. CAS no. 106-92-3 
	Acrylamides (1) e.g. Cas no. 79-06-1 
Polyalkyl ureas (0)	Benzotriazole-hindered phenols (0)
Thalidomide compounds (0)	Boron compounds (0)
Pyrroline esters, Pyrroline N-oxide esters, Pyrrole ester, Pyrrole alchohol (0)	Hindered amines (0)
Triazole antifungal analogues (0)	Nickel compounds-not evaluated
Retinoids and analogues (0)	Vinyl esters (0)

Isomers may have different receptor-binding affinity. Therefore, the risk assessor needs to evaluate whether isomers e.g. trans and cis, R and S isomers may have a different toxicological outcome because the isomers may bind differently to receptors and thus influence receptor-mediated pathways. The different functions of these specific isomers have shown their importance in drugs, agrochemicals and fragrances. The toxicological importance is less known except one important example being thalidomide (softenon).

The oral, dermal and inhalation bioavailability and the similarity between the target and the source chemicals can be addressed using physicochemical properties such as molecular weight, water solubility, log K_{ow} and vapour pressure, in case no experimental toxicokinetic data are available. In addition, the effects seen in *in vivo* testing can be used to show that the substance is systemically available. There can be effects

seen in the acute tests and the repeated-dose toxicity testing. In case the substance is a sensitiser, some dermal availability can also be expected.

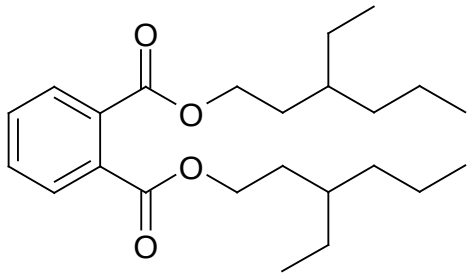
There is a wealth of expert judgment on the effects to be expected based on the bioavailability, reactivity and receptor binding of the source chemicals. A handful of pertinent references are described below. Janer et al (2007) have found that the NOAEL from a well-conducted 90-day study is sufficient to cover the male and female reproductive toxic effects and that a one- and two-generation studies may be superfluous. These papers were followed up with similar findings by Dang et al (2009) and Dent (2007).

Blackburn et al (2011) have shown a decision tree type of approach for reproductive and developmental toxicity. Quite a wide range of chemicals may be reproductive toxicants. For example, the decision tree indicates that metals and organophosphorus and phosphoramides may be reproductive or developmental toxicants. Also compounds with aromatic or heteroaromatic rings are an indicator, as well as cyclic rings with fused polycyclic nucleus. When using this decision tree the analogues or category members should fall into the same group. Whether the target and the source chemical(s) are reproductive or developmental toxicants depends on the information of the source chemical(s).

There are a variety of *in vitro* testing methods and batteries to assess reproductive and developmental toxicity, e.g. receptor-binding assays or those assays in use under the US EPA's EDSP (Endocrine Disruption Screening Program).

Besides the reproductive or developmental toxicity of the parent chemicals the toxicity of the metabolites may need to be assessed. The phthalates are a category where one of the metabolites causes the specific male fertility (Fabjan et al, 2006). A note has to be made because this male fertility effect is most apparent between the C4-C6 side chain. The very short and much longer side chains do not or much less show this effect.

Figure 7: An example of phthalates: Diethylhexylphthalate, known to cause effects to male fertility



A similarity in the toxicological profile can further support the justification of the read-across of reproductive and developmental toxicity. In case of cytotoxicity, this is probably more straight-forward compared to receptor-binding activity.

General considerations when performing read-across for reproductive and developmental toxicity

Reading across for reproductive and developmental toxicity faces similar challenges as with repeated-dose toxicity, in that they are complex endpoints and small variations in structure can lead to significant differences in reproductive or developmental toxicity. For example, consider 1-methoxypropan-2-ol and 2-methoxypropan-1-ol, the structural two isomers of propylene glycol methyl ether. The second is a developmental toxicant due to the formation of methoxypropanoic acid. The first is not capable of forming this metabolite due to its secondary rather than primary alcohol. Reading across from one isomer to the other is therefore inappropriate, however until data were generated on 2-methoxypropan-1-ol there was no evidence that it had a different developmental toxicity profile. Due to the potential complexity of the reproductive and developmental endpoints and the fact that a positive finding leads to a classification according to GHS, there is more conservatism in assessing read-across for these endpoints than others, at least from a regulatory perspective. Taking this into consideration, there is therefore potentially a higher burden of proof necessary to support read-across compared even to repeated-dose toxicity endpoints.

This raises the question about the data needed to support read-across for reproductive and developmental toxicity endpoints. A first consideration would be to generate on as many category members as possible the reproductive screening study (OECD 421, 1995). Consistency in the results from this study should give greater confidence in then reading across for the higher tier studies such as multi-generation studies and developmental toxicity studies. In addition to considering screening studies, more recently conducted repeated-dose toxicity studies often include assessments of reproductive organs (gross and histopathology). This information is also useful in demonstrating consistency in toxicity to reproductive organs across the group. For example, within the ethylene glycol ethers, ethylene glycol methyl ether expresses male reproductive toxicity through effects on the testes. It was possible to demonstrate the absence of this effect in other ethylene glycol ethers using reproductive screening studies and repeated-dose toxicity studies rather than perform multi-generational reproductive toxicity studies on each member.

When formulating a read-across argument for reproductive and developmental toxicity, it is important to also recognise that reading across positive findings is more easily accepted due to the conservatism in this approach. On the other hand, reading across an absence of developmental or reproductive toxicity would require a higher burden of proof, perhaps including reproductive screening studies on enough of the category members to demonstrate a consistent absence of effects. One can also utilise *in vitro* studies such as whole embryo culture, mouse limb-bud assays, *in vitro* testicular toxicity studies, or other available assays that could support the use of read-across without resorting to the conduct of several, animal intensive studies. However, due to their limited coverage of endpoints and varying states of validation, these are again more likely to be accepted as supporting evidence where they demonstrate existence rather than absence of an effect. Finally, as with the repeated-dose toxicity endpoint, (Q)SAR tools can be used to support read-across for reproductive and developmental toxicity endpoints. However, these tools are better utilised in demonstrating consistency across a group of substances rather than being used in isolation to predict this endpoint.

One very real challenge with using read-across for these endpoints within the context of REACH is that for the lower volume substances (10-100 tonnes) the only data requirement is for a reproductive/

developmental screening study. It is therefore important to understand what data could be used to support read-across for this endpoint without resorting to performing the reproductive/developmental screening studies on each category member.

6.6 Classification and labelling comparison

The use of read-across should not by default lead to a more conservative classification and labelling outcome. Rather, the read-across should in principle result in the same outcome as the source analogue(s). Exceptions might arise if the category is large and subcategories exist e.g. breakpoints due to water solubility for aquatic toxicity or differences in mode of action due to metabolism for systemic effects.

7. RECOMMENDATIONS

There are many endpoints where read-across can be applied and these range in complexity and sophistication from simple physicochemical and acute/local effects to repeated-dose/systemic and reproductive toxicity. This range of endpoints translates to a range in complexity of approaches that need to be developed, i.e. not a simple one size fits all. The foundation of many categories or read-across justification is that the substances are similar in structure (same functional groups) or have a common/shared metabolic pathway or precursors.

Data on toxicokinetics can be a key piece of evidence to support these justifications. For example, for the groups with common functional groups, the category relies on the fact that these substances is dealt with in a similar or predictable manner by the body, allowing one to use data from one substance to read-across to another with the same functional groups. Toxicokinetic data can allow demonstrating that the substances in the category are indeed metabolised in a similar manner, have similar absorption and excretion kinetics and similar distributions and therefore would be expected to have similar toxicological properties. These data can also aid in defining trends across a category for example, confirming that as molecular weight increases, bioavailability decreases, thus reducing the potential for systemic toxicity. Therefore, having this information has the potential to reduce the uncertainty in assessing read-across and reduce the need for intensive toxicological studies on every substance.

Where a category is defined by the metabolic pathway, the need for toxicokinetic data is self-evident and, in the majority of cases, without it one may not be able to prepare a sufficiently robust case to support the validity of the category. However, for the purposes of submitting a REACH registration dossier there is no mandatory requirement to perform a metabolism or toxicokinetic study. In some cases, there are toxicokinetic studies available. Where this is not the case, an assessment of toxicokinetics can be made using available toxicological studies, predictions based on (Q)SAR models, and/or conservative assumptions of parameters such as absorption. In the absence of a mandatory requirement for toxicokinetic information, there is often a reluctance to perform these types of studies due to their potential cost and complexity, particularly if radiolabelled test material is required.

Given the potential utility and importance of toxicokinetic data in the hazard characterisation process, planning of higher tier studies or in supporting categories and read-across, rather than conducting bespoke toxicokinetic studies on each substance, consideration should be given to including a toxicokinetic element in standard toxicological studies. Saghir et al (2012) and Creton et al (2012) recently published overviews of the possible inclusion of toxicokinetic parameters in standard guideline studies from sub-acute to chronic repeated-dose toxicity studies, developmental and reproductive toxicity studies. This program is currently focused on toxicological studies assessing plant protection products; however it could be utilised when testing other chemicals, for example, those subject to REACH. The addition of toxicokinetic parameters to standard toxicological studies can be done relatively inexpensively and does not require the use of radiolabelled material by default. The measurements can take place during the range finding of a study using a limited number of animals, hence should not result in an increase in the number of animals used per study.

In a situation where multiple members of a category require a base set of toxicological studies, the addition of toxicokinetic parameters to those studies could be done whenever possible. In addition, and from a

regulatory perspective, where the validity of read-across is challenged, the possibility of generating additional toxicokinetic information in a range finding study could be considered before ultimately rejecting the read-across justification. Adopting this approach allows a registrant to conduct the range finding study for a potential higher tier study while generating toxicokinetic information. This data may provide the additional information necessary to support the read-across justification while minimising the use of animals.

There are currently two examples of where this approach is being taken within industry to support the use of read-across. In the first example, a glycol ether undergoing an OECD 422 (1996) combined reproductive/developmental and repeated-dose toxicity study has been assessed for toxicokinetic parameters during the range finding stage of the study. These data are then used to demonstrate the substance has a common metabolic pathway to a data-rich glycol ether, supporting the use of read-across. In the second example, an alcohol and a ketone assessed in a limited *in vivo* toxicokinetic study using a small group of animals to demonstrate that they share the same metabolic pathway, such that they 'inter-convert' so that exposure to one results in systemic exposure to the other.

In brief:

- Toxicokinetic data are valuable in characterising hazards, designing future studies and supporting read-across.
- Toxicokinetic data can be collected during standard toxicological studies rather than relying on bespoke toxicokinetic studies.
- Toxicokinetic parameters can be collected relatively inexpensively, with minimum impact on the number of animals used.

8. RESEARCH NEEDS/ACTIVITIES

There are several reasons why read-across has been limited to the analogue approach and to small chemical categories of structurally similar substances. To begin with, the desire to minimise uncertainties associated with application of a read-across resulted in selecting a handful of closely related analogues. Moreover, there remains a mixed understanding of the mechanisms and modes of action for all (eco)toxicology endpoints. Certain endpoints are better understood than others and this is mirrored both in the extent to which there have been some insights derived from (Q)SAR approaches which characterise the context of similarity as well as the availability of *in vitro* assays for these endpoints. For example, validated *in vitro* alternatives exist for skin/eye irritation. Rulebase systems also exist for irritation endpoints, which enables a read-across to be more readily structured and substantiated. Ecotoxicity endpoints have been well characterised in terms of modes of action, the Verhaar scheme (Verhaar et al, 1992; 1995; 2000) being the most notable example to illustrate how structural insights can be encoded into rules to group structurally-related substances together.

The OECD (Q)SAR Toolbox, with its many profiling schemes in effect encodes relevant MOA information that can be used to group related substances together. Other expert systems and rulebase schemes previously discussed essentially provide that similarity context. However as gleaned from the previous sections, these work to an extent for only a handful of endpoints where a clear link between structure and endpoint can be made – the MIE. This works well for more 'local' or 'acute' endpoints. For genotoxicity endpoints such as Ames or *in vitro* chromosomal aberration, skin sensitisation, skin/eye irritation, acute aquatic toxicity, a clear relationship can be drawn between structure and effect. This is mirrored by the available (Q)SAR and the available profiling tools. The disconnection and difficulty remains on how to robustly characterise and read-across for more challenging endpoints such as repeated-dose toxicity, reproductive/developmental endpoints. Whilst some (Q)SAR exist for these type of endpoints, these are either local (Q)SAR with limited scope of focusing in on small chemical classes or statistical models that summarise a complex endpoint with a binary outcome and use topological information (typically) to identify a statistical correlation with a training set of chemicals. These types of models do little to inform MOA and only serve to provide a prediction based on statistical correlation. In these cases, toxicokinetic studies plus limited *in vivo* testing is still proposed to formulate as far as possible a WoE approach. The ECETOC TR 109 report summarises state-of-the-art methods that can also be used to further substantiate read-across regarding systemic endpoints (ECETOC, 2010).

Toxicity Testing in the 21st Century (Tox21) shapes a new paradigm for the way in which toxicity testing will be carried out in the future (NRC, 2007). Instead of high dose toxicity testing in animals where there is little understanding about the mode or mechanism of action, the shift will be towards pathway approaches. These could be cellular response pathways, that, when sufficiently perturbed, are expected to result in adverse mammalian, ecotoxicological and environmental effects. As Tox21 evolves, the type of toxicity testing carried out and its interpretation to inform read-across approaches will be significantly different.

Ankley et al (2010) broadened the scope of pathways to define the broader construct of AOP as representing *"existing knowledge concerning the linkage between the molecular initiating event (MIE) and an adverse outcome at the individual or population level"*. By definition, an AOP spans multiple levels of biological organisation. An AOP links a molecular initiating event to an adverse outcome. MIE was defined as the initial point of chemical-biological interaction within the organism that starts the pathway.

Thus, an AOP delineates the documented, plausible and testable process by which a chemical induces molecular perturbations and the associated biological responses which describe how the molecular perturbations cause effects at the sub-cellular, cellular, tissue, organ, whole animal and (when required) population level of observation. The pathway approach is based on the concept that toxicity results from a chemical first reaching and then interacting with an initial key target (e.g. membrane, receptor) in the organism; this is defined as the MIE. Further to this, primary interaction begins a series of events that can individually be documented and tested, resulting in an adverse outcome (e.g. reproductive failure, neurotoxicity). Several pathways can result in the same adverse outcome, and each constitutes an individual AOP. OECD organised a workshop in 2010 on using mechanistic information in forming chemical categories (OECD, 2011); to discuss the way in which AOPs could be used for grouping chemicals. The report provides important definitions (e.g. molecular initiating event, toxicity pathway, mode of action, AOP, source to outcome pathway), as well as case studies on proposed AOPs.

Integrating knowledge of how chemicals interact with biological systems (i.e. the molecular initiating event) with knowledge of the responses at increasing levels of biological complexity should facilitate the derivation of chemical categories that can make robust predictions for longer-term effects. Thus, chemicals could be conceivably grouped according to their ability to trigger the same MIE of following key events. Importantly, a full pathway from initial molecular initiating event to the final adverse outcome need not be described for the purposes of being able to build a chemical category. But for the purposes of minimising uncertainties or building more confidence, more information down the pathway and the extent to which experimental data can be generated to substantiate the pathway downstream are helpful to establish the causal links between the Molecular Initiating Event (MIE) or Key events (KE).

Results from molecular screening and genomics methods such as those which are being generated as part of the ToxCast programme could be anchored in the context of a given AOP(s) to ensure that the data generated is being interpreted in the appropriate context and so that the grouping is done with the necessary information to substantiate the AOP. For example, oestrogen receptor (ER) binding can be used to screen chemicals for the potential to be reproductive toxicants via ER-binding. In this case, the molecular initiating event (MIE) is ER-binding and the AO is reproductive toxicity.

The AOP for skin sensitisation provides an illustration (OECD, 2012c) of how an AOP might be structured.

Recently the OECD described a work programme for AOPs under the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) and the Task Force of Hazard Assessment (TFHA); a call was made for Member Countries to propose AOPs that could be developed as part of this effort for which their application could be for IATA (Integrated Assessment Testing frameworks) and for building categories.

As part of this work programme, activities will be focused on developing a library of AOPs, a library of MIEs and a library of key endpoints of regulatory concern. As part of the first activity, OECD is partly funding a repository where AOP information can be stored, peer reviewed and discussion can be potentially managed. The repository is known as Effectopedia (www.effectopedia.org), a Wikipedia type framework that enables AOPs to be developed and modified as needed (OECD, 2011).

At the current time, read-across is likely to be limited to small categories or analogue approaches to compensate for a lack of understanding about certain endpoints or the MOA of specific chemicals. In the near term, as more emerging technologies come into routine use and as the library of repositories (likely through Effectopedia) will provide a means/anchor for MIEs and other KE such that grouping of substances can be robustly managed for both complex endpoints and for larger numbers of substances. In time, the insights and learning derived from generating data to substantiate MIE or KE events might be sufficient and conducive to developing specific profilers that can be used in place of generating the information experimentally.

ABBREVIATIONS

ADA	Alkyldimethylamine
ADAO	Alkyldimethylamine oxide
ADME	Absorption, distribution, metabolism and excretion
AIM	Analog identification methodology
AOP	Adverse outcome pathway
ARF	Analogue reporting format
ASTM	American Society for Testing and Materials
BAF	Bioaccumulation factor
BCF	Bioconcentration Factor
BfR	German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung)
BMF	Biomagnification Factor
BTEX	Benzene, toluene, ethylbenzene, and xylenes
CAS	Chemical abstracts service
CDAT	Chemical data access tool
CRAFT	Chemical reactivity and fate tool
CRF	Category reporting format
DART	Decision Analysis by Ranking Techniques
DEGBE	Diethylene glycol butyl ether
DEGEE	Diethylene glycol ethyl ether
Derek	Deductive estimation of risk from existing knowledge
DNA	Deoxyribonucleic acid
DNEL	Derived no effect level
DPRA	Peptide reactivity assay
DSL	Canadian domestic substances list
DSSTox	Distributed structure-searchable toxicity
EBA	Exposure-based adaptation
EBW	Exposure-based waiving
EC	European Commission
ECB	European Chemicals Bureau
ECHA	European Chemicals Agency
ECVAM	European Centre for the Validation of Alternative Methods
EDSP	Endocrine disruption screening program
EGEE	Ethylene glycol ethyl ether
EINECS	European INventory of Existing Commercial chemical Substances
EPA	Environmental Protection Agency
EPI	Estimation Programs Interface
ESIS	European chemical Substances Information System (ECB)
EU	European Union

FDA	Food and Drug Administration (US)
GC	Gas chromatography
GPMT	Guinea pig maximisation test
h-CLAT	Human cell line activation test
HC	High-content
HLC	Henry's Law constant
HMWPE	High molecular weight phthalate esters
HPV	High production volume
HPVIS	High production volume information system
HT	High-throughput
ICCA	International Council of Chemical Associations
IGC_{50}	50% inhibitory growth concentration
ILSI	International Life Sciences Institute
INChI	International Chemical Identifier
ITS	Integrated (Intelligent) testing strategy
JRC	Joint Research Centre
KE	Key event
kM	Biotransformation rate
K_{OA}	Octanol-air partition coefficient
K_{OC}	(Soil adsorption) coefficient of organic compounds
Lazar	Lazy structure-activity relationships
LC_{50}	Lethal concentration to 50% of the test animals
LD_{50}	Lethal dose to 50% of the test animals
LLNA	Local lymph node assay
LOAEL	Lowest observed adverse effect level
log K_{ow}	Log of the octanol-water partition coefficient
LRI	Long-range Research Initiative
MAA	Metacrylic acid
MCASE or MC4PC	Multiple Computer Automated Structure Evaluation/MCASE for Windows Personal Computers
MDA	Methylenediphenyl diamines
MDI	Methylenediphenyl diisocyanates
MEP	Ministry of Environmental Protection
MIE(s)	Molecular initiating event(s)
MMA	Methyl methacrylate
MOA	Mode of action
MRDD	Maximum recommended daily dose
MTD	Maximum tolerated dose
MUSST	Myeloid U939 skin sensitisation test
MW	Molecular weight

NCP	New chemicals program
NCTT	National Center for Computational Toxicology (US EPA)
NOEC	No observed effect concentration
NOAEL	No observed adverse effect level
NTP	National Toxicology Program
OECD	Organisation for Economic Cooperation and Development
PAH	Polycyclic aromatic hydrocarbon
PBPK	Physiologically-based pharmacokinetic modelling
PBT	Persistence, bioaccumulation, toxicity
PCA	Principal components analysis
PMN	Pre-manufacture notices
PNEC	Predicted no effect concentration
QAAR	Quantitative activity-activity relationship
QSAAR	Quantitative structure-activity-activity relationship
QMRF	(Q)SAR model reporting format
QPRF	(Q)SAR prediction reporting format
(Q)SAR	(Quantitative) structure activity relationship
(Q)SBR	Quantitative structure-biodegradation relationship
RAAF	Read-Across Assessment Framework
RAEG	Read-Across Expert Group
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RIP	REACH Implementation Project
SA	Structural alerts
SAR	Structure activity relationship
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SIAM	SIDS (Screening Information Data Set) Initial Assessment Meeting
SIDS	Screening Information Data Set (OECD)
SMILES	Simplified molecular input line entry system
SOM	Self-organising map
SRC	Syracuse Research Corporation
TEGBE	Triethylene glycol butyl ether
TEGEE	Triethylene glycol ethyl ether
TEGME	Triethylene glycol methyl ether
T.E.S.T.	Toxicity Estimation Software Tool
TetraEGME	Tetraethylene glycol methyl ether
TG	Test guideline
TGD	Technical Guidance Document
TIMES	Tissue MEtabolism Simulator
TMF	Trophic magnification factor

TSCA	Toxic Substances Control Act
TTC	Threshold of toxicological concern
UBA	German Federal Environment Agency (Umweltbundesamt)
US EPA	United States Environmental Protection Agency
UVCB(s)	Substance(s) of unknown or variable composition, complex reaction products or biological materials
VLCFA	Very long-chain fatty acid
WoE	Weight of evidence
WSol	Water solubility

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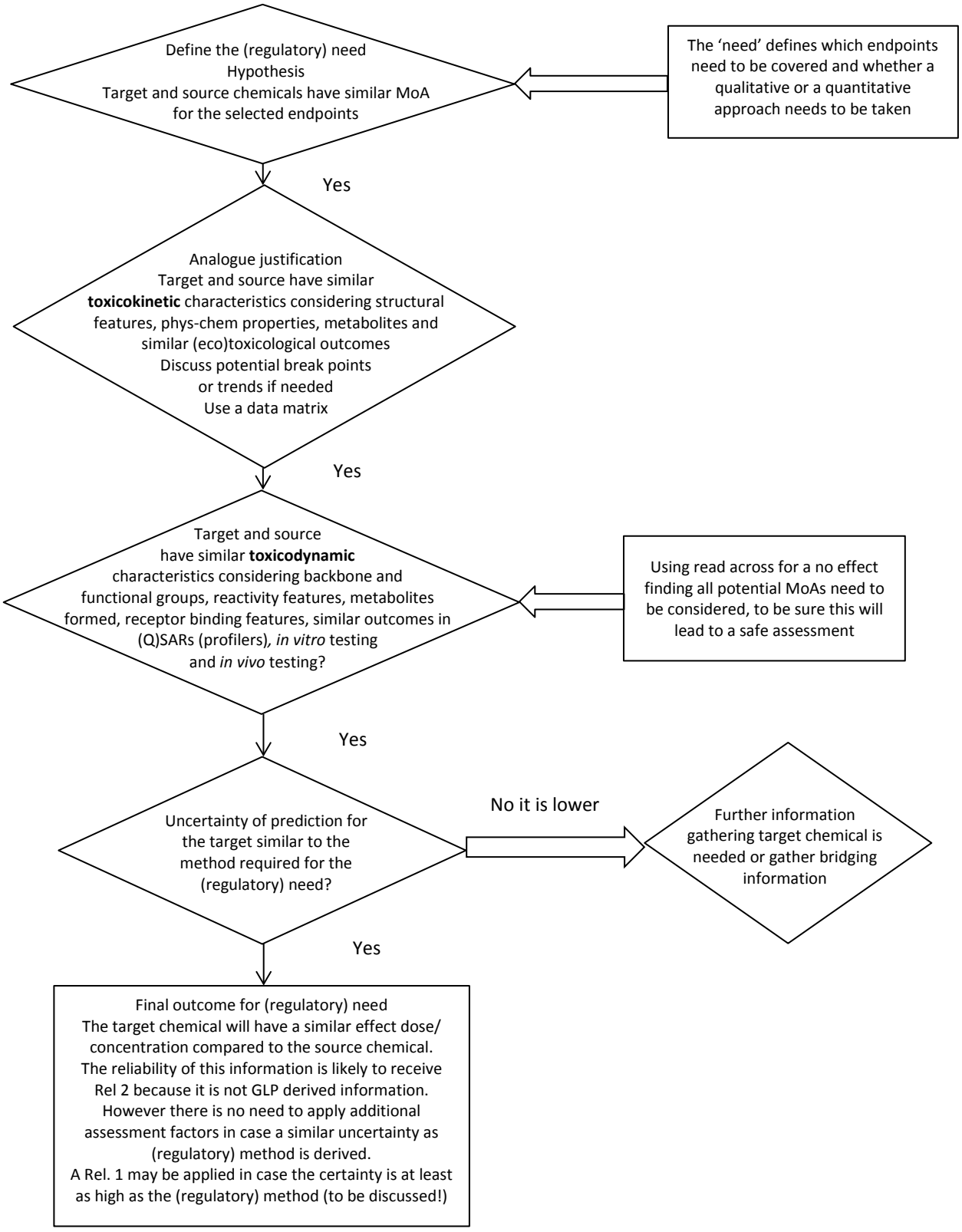
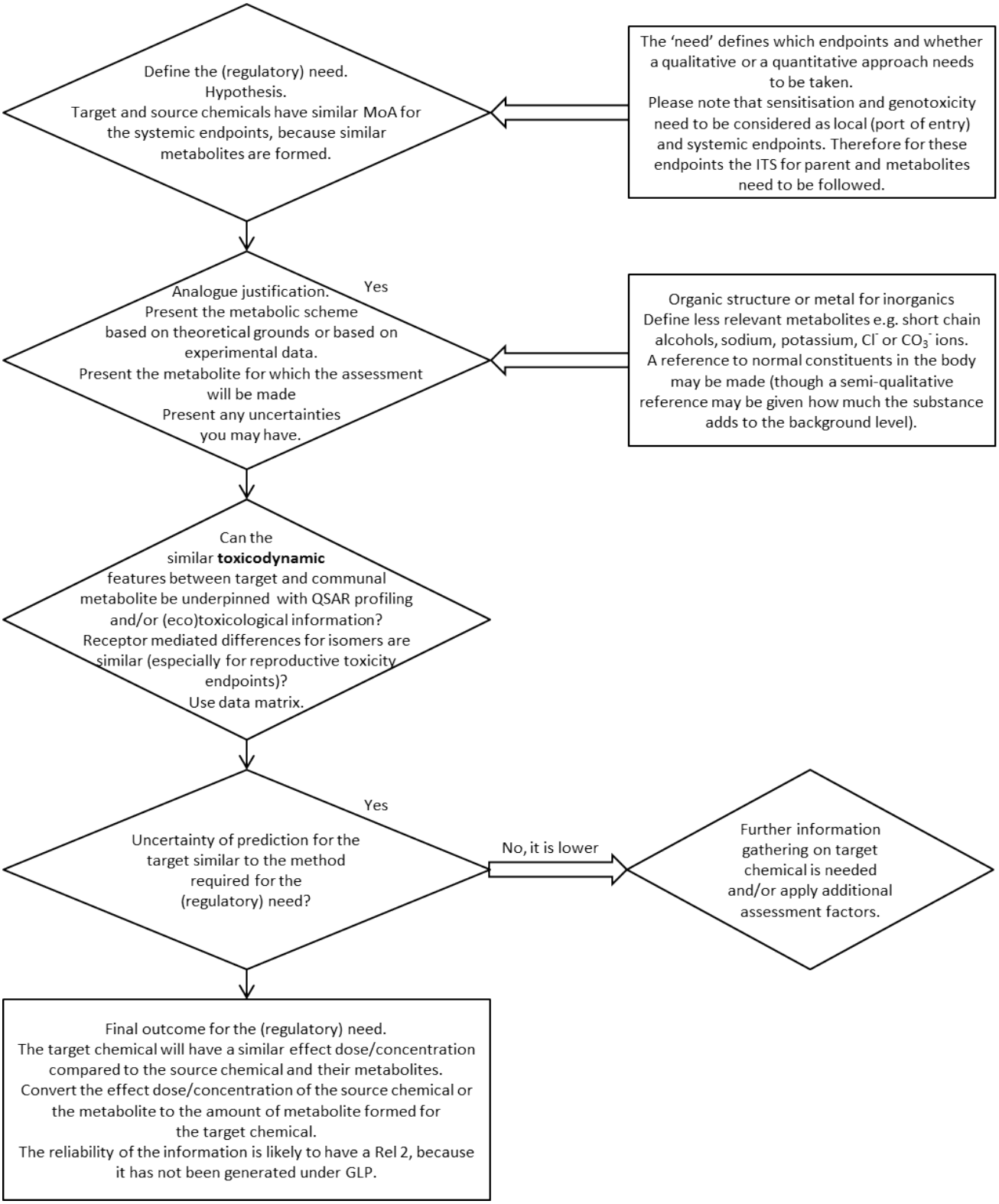
APPENDIX A: WORKFLOWS

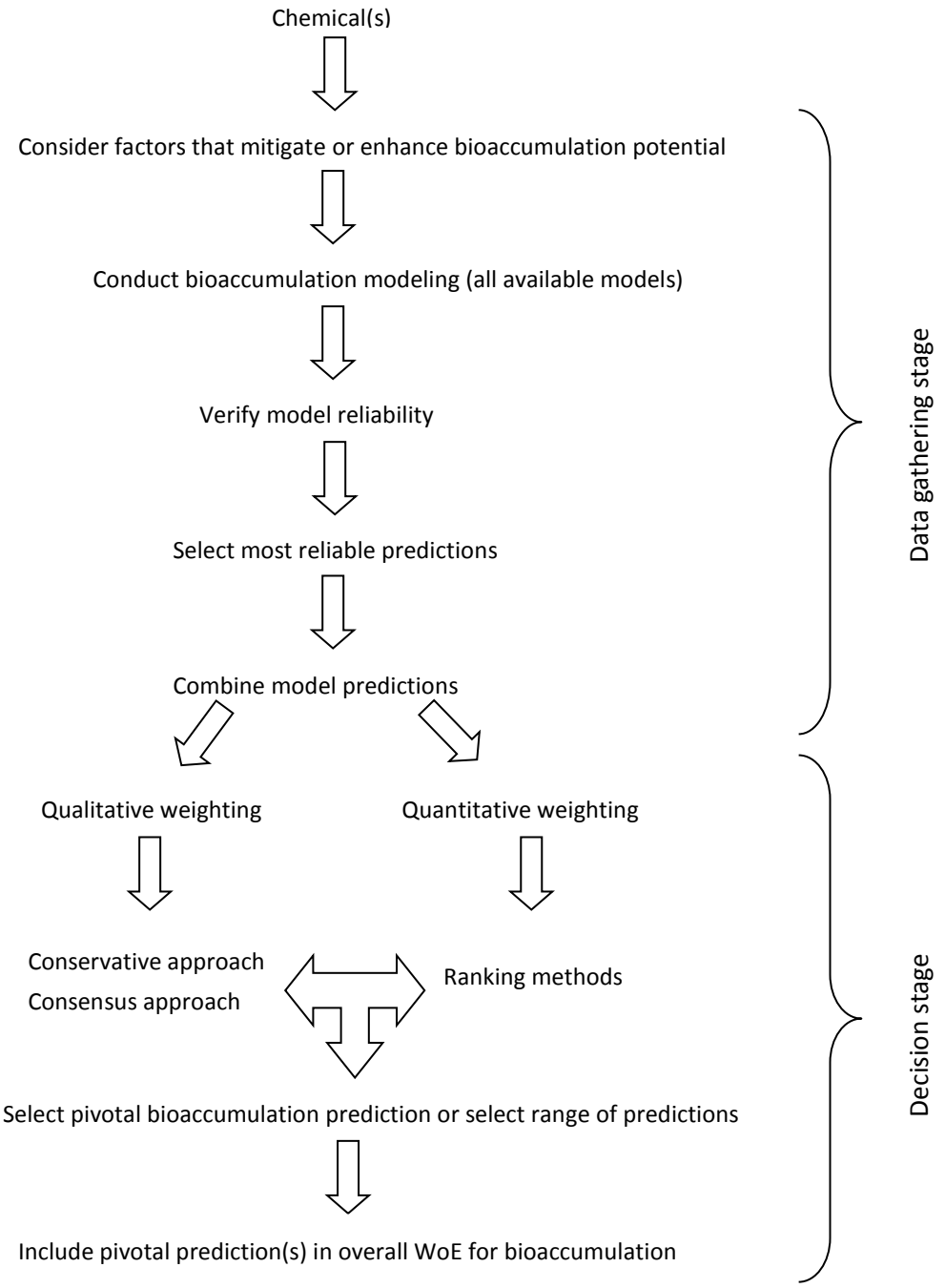
General workflow characterising the WoE associated with analogue approaches for parent substances and their metabolites



General workflow characterising the WoE for predicting the target toxicity using a metabolic approach



Workflow characterising the WoE for bioaccumulation (adapted from Nichols et al, 2009)



APPENDIX B: CASE STUDIES

B.1. Cycla-esters

B.1.1 Using read-across for assessing the skin sensitisation potential of Cyclacet and Cyclaprop being Cycla-esters

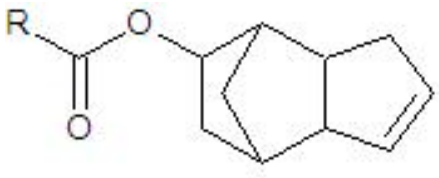
1. Hypothesis for the analogue approach

Cyclacet and Cyclaprop are not expected to be sensitisers because the longer-chain analogues Cyclobutanate and Cyclabute have not shown sensitising potential in well-performed guinea pig maximisation tests (GPMT). These source chemicals Cyclobutanate and Cyclabute are sufficiently similar to read across towards Cyclacet and Cyclaprop.

2. Target and source chemical

The Cycla-esters have three fused rings with an unsaturated bond in the right ring. On the left ring an alkyl chain is attached to the ester bond.

Figure B1-1: Cycla-ester. R is an alkyl chain C1-C3



The source chemicals are e.g. butyl-esters while the target chemicals have an ethyl (acetic) or a propyl-ester. These differences in 2-D structures of the source chemicals are not expected to behave differently compared to the targets because the alkyl side chains (butyl versus ethyl/propyl) are not expected to influence significantly the reactivity of the ester bond which is relevant for skin sensitisation properties.

3. Purity/impurities

The purity and impurities of the source chemicals do not indicate sensitisation potential. The impurities are all below 1%. Also the purity and impurities of the target chemical do not indicate sensitisation potential and are below 1%.

4. Analogue approach justification

According to Annex XI, 1.5 a read-across approach can be used to fill the data gap when certain criteria are fulfilled. The fulfilment of these criteria are discussed below. The information from the REACH technical guidance document R.6 are used for this assessment as well as ECHA's Practical guide 6 on category and read-across approaches (ECHA REACH TGD; ECHA, 2009).

Quality of the experimental data of the analogues

The source chemicals Cyclabute and Cyclobutanate have been tested in well-conducted GPMT (OECD TG 406 under GLP). The challenge concentrations used were 50 and 100%. The test results receive reliability 1.

Toxicokinetics

The source chemicals and the target chemicals indicate similarity in toxicokinetic behaviour based on the molecular weight (< 250), physical form (all are liquids), vapour pressures (1-10 Pa) as is shown in the data matrix.

It can be seen that the water solubility of the source chemicals, which contain the butyl esters, will be somewhat lower and the log K_{ow} somewhat higher compared to the target chemicals which contain the ethyl and propyl ester. For the toxicokinetic behaviour of both chemicals, these physicochemical differences are expected to be minimal considering absorption via the dermal route, an important parameter for skin sensitisation potential. The physicochemical properties of these chemicals are still within the range for expected ready absorption. Therefore no differences are expected in sensitisation potential considering these kinetic properties.

Reactivity towards proteins

Expert judgment

Esters containing carbon, hydrogen and oxygen atoms without adjacent activating groups such as double bonds are not expected to be sufficiently reactive to proteins to cause skin sensitisation.

(Q)SAR modelling

The (Q)SAR modelling as such is not used for predictivity but it is used for showing that the Cycla-esters have the same skin sensitisation profile according to these models.

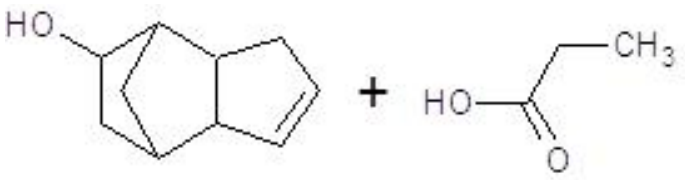
The Cycla-esters do not fall into one of the reactivity mechanisms discussed by Aptula et al (2005) and Aptula and Roberts (2006), which are included in the OECD (Q)SAR Toolbox and Toxtree software programs. These programs show a similar pattern for all Cycla-esters and for the Cycla-alcohol, which is one of the cyclic esters. In the Toolbox there is one profiler indicating activity. This is considered a false positive prediction because the source chemicals are not positive (see B.1.5 for an overview of the profiling of the Toolbox). In addtion the Cycla-esters are out of the applicability domain of this structural alert. The Caesar model

predicts similar activity for the Cycla-ester and its alcohol. The positive prediction is considered a false positive because the functional groups in the presented analogues are aldehydes and/or have conjugated bonds, which are structural features not related to the Cycla-esters.

Non-sensitising common breakdown products or metabolites

In the case of esters, an acid and an alcohol are the metabolites. A (qualitative) assessment of both metabolites is needed. The alcohol is generally considered less reactive considering skin sensitisation compared to the ester bond and because the parent substance is not expected to be a sensitiser; also the metabolite is not a sensitiser. This again can be underpinned by (Q)SAR models (data not shown). The acid is expected to be somewhat more reactive and may cause (slight) skin irritation but acids as such rarely cause skin sensitising e.g. carboxylic acids such as ethyl-, propyl-, and butyl-acids.

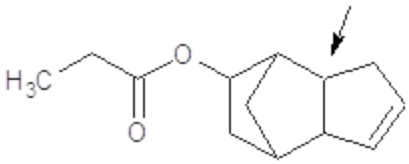
Figure B1-2: Metabolites of the Cycla-esters



The OECD (Q)SAR Toolbox and Toxtree present the absence of skin sensitisation for both the alcohol and the acid. Caesar shows ‘active' for sensitisation using the same chemicals as for the Cycla-esters and their alcohol (data not shown).

The Cycla-esters are not expected to auto-oxidise and form peroxides in a significant way. These chemicals have one spot where potential auto-oxidation can occur according to the skin metabolism profiler of the OECD (Q)SAR Toolbox. This spot is however a secondary carbon at which it is quite unlikely that peroxide formation occurs because such a secondary alcohol is insufficiently electronegative and insufficiently stable in contrast to peroxide formation to a tertiary carbon. Therefore this prediction of the Toolbox is applicable in a limited way to these esters. From experience in handling the substance no such peroxide formation is expected and no anti-oxidants are added to prevent such oxidation. Therefore the auto-oxidation of the Cycla-esters does not need to be considered.

Figure B1-3: A spot in the Cycla-ester structure which may auto-oxidise is indicated with an arrow and the potential metabolite is shown



Similarities in results for toxicological endpoints between the target and the source chemical(s) to support read-across for sensitisation

As is presented in the data matrix (B.1.4), the acute oral and dermal toxicity data show limited acute toxicity for both the source and the target chemicals. The source and target chemicals show slight skin and eye irritation indicating similar reactivity. All Cycla-esters show negative results in the Ames tests. In the 28-day repeated-dose toxicity study of Cyclabutanate no effects were seen at > 1000 mg/kg bw and for Cyclacet no repeated-dose toxicity was seen in the reproductive/developmental toxicity screening test, also indicating the low reactivity potential. The limited toxicological effects in these endpoints further support the limited reactivity and the non-sensitisation potential of the Cycla-esters.

Uncertainty of the prediction

The predictions of the absence of skin sensitisation for the target chemicals have a high certainty because of close similarity between the source and the target chemicals. The differences in the alkyl chain, one or two methyl groups, will not have an impact on the skin sensitisation potential because of absence of activating fragments. The skin sensitisation information from the source chemicals are high quality GPMT (reliability 1). The (Q)SAR predictions which are in the applicability domain of the model predict the absence of skin sensitisation. Though the OECD (Q)SAR Toolbox, Toxtree may in an only limited way fulfil principal 4 on predictivity of the OECD principles on the validation of (Q)SARs on the absence of skin sensitisation, the MoA for skin sensitisation are well established and therefore these predictions can be taken into account.

5. Data matrix

See B.1.4.

6. Skin sensitisation prediction for Cyclacet and Cyclaprop

For Cyclabute and Cyclobutanate (butyl side chains), well-conducted GMPT are available (reliability 1). The substance is a liquid and concentrations of 50% and 100%, respectively were used for challenge. This indicates that Cyclacet (ethyl side chain) and Cyclaprop (propyl side chain) are not skin sensitisers either.

Final conclusion on hazard, C&L, DNEL and risk characterisation

Cyclacet and Cyclaprop and their metabolites have no indication for skin sensitisation. Classification and labelling is not needed and therefore a DNEL does not need to be derived and a risk characterisation is not needed either.

7. Respiratory sensitisation

The presented Cycla-esters have no indication for skin sensitisation and are therefore not respiratory sensitisers either (REACH technical guidance document: Scheme of R7A, Fig. 7.3-2). In addition, the Cycla-esters have a low volatility (< 10 Pa).

B.1.2 Using read-across for assessing the repeated-dose toxicity of Cyclaprop from other Cycla-esters

1. Hypothesis for the analogue approach

Cyclaprop has similar repeated-dose toxicity compared to the other Cycla-esters resulting in a similar no observed adverse effect level(NOAEL), which is 1000 mg/kg bw, being the highest dose tested. Cyclaprop has a similar backbone and a similar functional group as all Cycla-esters (Figure B1-1). The one methyl longer-chain analogue Cyclobutanate has not shown any toxicologically relevant repeated-dose toxicity in the 28-day toxicity tests (oral route: OECD TG 407). In addition, no parental toxicity was seen in a Reproscreen study (OECD TG 421) with Cyclacet which is a one methyl group shorter-chain analogue. The information from these source chemicals, Cyclacet and Cyclobutanate, is sufficiently reliable and the substances are sufficiently similar to read across towards Cyclaprop.

2. Target and source chemical

The Cycla-esters have three fused rings with an unsaturated bond in the right ring. On the left ring an alkyl chain is attached to the ester bond (See Figure B1-1).

The source chemicals are an ethyl and a butyl-ester while the target chemical has a propyl-ester. These differences in chemical structures of the source chemicals are not expected to behave differently compared to the target because the alkyl side chains (ethyl and butyl versus propyl) are not expected to influence significantly the reactivity of the ester bond which is relevant for repeated-dose toxicity properties.

3. Purity/impurities

The purity and impurities of the source chemicals do not indicate sensitisation potential. The impurities are all below 1%. Also the purity and impurities of the target chemical are below 1%.

4. Analogue approach justification

According to Annex XI 1.5, a read-across approach can be used to fill the data gap when certain criteria are fulfilled. The fulfilment of these criteria are discussed below. The information from the REACH technical

guidance document R.6 is used for this assessment as well as ECHA's Practical guide 6 on category and read-across approaches (ECHA REACH TGD; ECHA, 2009).

Quality of the experimental data of the analogues

See B.1.4 for an overview of the experimental repeated-dose toxicity data.

The source chemicals Cyclacet and Cyclobutanate have been tested in reliable OECD TG 421 and OECD TG 407 tests up to 1000 mg/kg bw, respectively. Therefore these data have limited uncertainty and can be used in an analogue approach.

Toxicokinetic

The source chemicals and the target chemicals indicate similarity in toxicokinetic behaviour based on the molecular weight (< 250), physical form (all are liquids), and vapour pressures (1-10 Pa) as is shown in the data matrix (B.1.4). It can be seen that the water solubility of the source chemicals containing butyl esters will be somewhat lower, and the log K_{ow} somewhat higher, compared to target chemicals which contain a propyl ester. For the toxicokinetic behaviour of the target chemicals, these physicochemical differences are expected to be minimal considering absorption via the oral, inhalation and dermal route, because these properties of the chemicals are still within the range for expected ready absorption. For repeated-dose toxicity, the kinetic behaviour will be sufficiently similar to read-across between the Cycla-esters.

Reactivity

Experimental data

The source chemical Cyclobutanate has been tested in well-conducted 28-day repeated-dose toxicity test (OECD 407) and shows no toxicologically relevant reactivity in the repeated-dose toxicity testing: NOAEL is 1000 mg/kg bw. The test results receive reliability 1. The target chemical Cyclaprop is a propyl ester while the source chemical is a butyl ester. This difference in chain length is not expected to change the reactivity of the ester bond and will thus have the same NOAEL in a repeated-dose toxicity study. In addition, Cyclacet (ethyl ester) did not show any relevant repeated-dose toxicity in the Reproscreen study the OECD TG 421 (NOAEL 1000 mg/kg bw for repeated-dose and reproductive toxicity) which further supports the expected limited repeated-dose toxicity for the target chemical Cyclaprop.

Expert judgment

The reactivity of these Cycla-esters is low as can be seen in the experimental data available. The double bond in the last ring does not show any reactivity considering repeated-dose toxicity. The double bond in this ring can be at the 5- and 6-yl position but this is not expected to influence the results. This double bond is similarly present in all Cycla-esters and therefore will not change the outcome of the repeated-dose toxicity.

(Q)SAR modelling

The Cycla-esters all fall into the same Cramer Class III, because they contain the ester bond. The Cycla-esters show a similar (non) reactivity profile for skin sensitisation and genotoxicity which further supports the hypothesis that Cycla-ester information can be used to read across repeated-dose toxicity from Cyclabutanate to Cyclaprop (OECD (Q)SAR Toolbox, 2012, see B.1.5). Some alerts are fired for all Cycla-esters further confirming that these are in the same structural activity group. The experimental testing information shows that the substances have limited toxicity. Also ECOSAR models identify all four esters in the ester class. Derek Nexus does not identify any structural alert for the Cycla-esters.

Common breakdown products or metabolites

In the case of esters, an acid and an alcohol are the metabolites (See Figure B1-2). These metabolites have been assessed during testing of the parent chemicals. No additional experimental information on the Cycla-alcohol is available. This alcohol falls into the Cramer Class I being less reactive compared to Class III. The acids formed are acetic, propyl and butyl acids and are common acids in the body.

Similarities in results for toxicological endpoints between the target and the source chemical(s)

As is presented in the data matrix (B.1.4), the acute oral and dermal toxicity data show limited acute toxicity for both the source chemicals and the target chemicals. The source and target chemicals show slight skin and eye irritation, which generally do not warrant C&L. The negative genotoxicity profile is also similar between the source and the target chemicals. For all Cycla-esters the Ames test is negative. As has been presented earlier, Cyclabutanate and Cyclacet have limited systemic toxicity. In the 28-day repeated-dose toxicity study, a NOAEL of > 1000 mg/kg bw was established. For Cyclacet, no repeated-dose toxicity was seen in the reproductive/developmental toxicity screening test. Therefore a similar result is expected for Cyclaprop.

Uncertainty of the prediction

The NOAEL predictions for the repeated-dose toxicity have a high certainty because of close similarity between Cyclaprop, the target chemical and the source chemicals. The one methyl group differences in the alkyl chain are not expected to have an impact on the repeated-dose toxicity potential because of absence of activating fragments. All these chemicals are expected to metabolise into the respective Cycla-alcohol and their acids. Therefore, the reliability of the 28-day NOAEL prediction for Cyclaprop is considered to be 1.

5. Data matrix

See B.1.4.

6. Repeated-dose toxicity prediction for Cyclaprop

For the source chemical Cyclobutanate (butyl side chain) a well-conducted OECD TG test 407 is available and for Cyclacet (ethyl side chain) a well-conducted Reproscreen test (OECD TG 421) (reliability 1) showing absence of repeated-dose toxicity at 1000 mg/kg bw, the highest dose tested). This means that a similar result for Cyclaprop can be anticipated.

Final conclusion on hazard, C&L, DNEL and risk characterisation

Cyclaprop and its metabolites have no indication for repeated-dose toxicity and the derived NOAEL for Cyclaprop is set at 1000 mg/kg bw for sub-acute exposure (28-day). A DNEL for oral, dermal and inhalation route can be based on this information. Classification and labelling are not needed for this endpoint. A risk characterisation will be performed because the Cycla-esters are classified for environmental toxicity. This risk characterisation will further ensure safe use considering repeated-dose toxicity. In addition, information from a 90-day repeated-dose toxicity study will become available for Cyclacet in 2012/2013. This information will also be used to see whether the AF to account from sub-acute to sub-chronic effects is sufficient.

B.1.3 Using read-across from Cycla-esters to assess the reproductive and developmental toxicity potential of Cyclaprop

1. Hypothesis for the analogue approach

Cyclaprop has similar reproductive and developmental dose toxicity compared to Cyclacet and Cyclabutanate resulting in a similar NOAEL because the ethyl- and the butyl-chain analogues have not shown any reproductive or developmental toxicity in the 28-day toxicity tests (OECD TG 407) and no fertility and developmental toxicity was seen in a Reproscreen study (OECD TG 421) with Cyclacet which is a one methyl smaller-chain analogue. The information from these source chemicals Cyclacet and Cyclobutanate are sufficiently similar to read-across towards Cyclaprop.

2. Target and source chemical

The Cycla-esters have three fused rings with an unsaturated bond in the right ring. On the left ring an alkyl chain is attached to the ester bond (See Figure B1-1).

The source chemicals are ethyl and butyl-esters while the target chemical is a propyl-ester. These differences in chemical structures of the source chemicals are not expected to behave differently compared to the target because the alkyl side chains (ethyl and butyl versus propyl) are not expected to influence significantly the reactivity of the ester bond of the receptor binding which is relevant for reproductive and developmental toxic properties.

3. Purity/impurities

The purity and impurities of the source chemicals do not indicate sensitisation potential. The impurities are all below 1%. Also the purity and impurities of the target chemical are below 1%.

4. Analogue approach justification

According to Annex XI 1.5 a read-across approach can be used to fill the data gap when certain criteria are fulfilled. The fulfilment of these criteria are discussed below. The information from the REACH guidance document R6 are used for this assessment as well as ECHA's Practical guide 6 on category and read-across approaches (ECHA REACH TGD; ECHA, 2009).

Quality of the experimental data of the analogues

See B.1.4 for an overview of the experimental data.

The two source chemicals Cyclacet and Cyclobutanate have been tested in reliable OECD TG 421 and OECD TG 407 tests up to 1000 mg/kg bw. Therefore these data have limited uncertainty and can be used in an analogue approach.

Toxicokinetic

The source chemicals and the target chemicals indicate similarity in toxicokinetic behaviour based on the molecular weight (< 250), physical form (all are liquids), vapour pressures (1-10 Pa) as is shown in the data matrix (see B.1.4). It can be seen that the water solubility of the source chemicals containing butyl esters will be somewhat lower, and the log K_{ow} somewhat higher, compared to target chemicals which contain a propyl ester. For the toxicokinetic behaviour of the target chemicals, these physicochemical differences are expected to be minimal considering absorption via the oral, inhalation and dermal route, because these properties of the chemicals are still within the range for expected ready absorption. Therefore the minimal differences in the kinetics do not influence the reproductive and developmental toxicity between the target and the source chemicals.

Reactivity and receptor binding

Experimental data

The source chemicals Cyclacet and Cyclobutanate have been tested in well-conducted OECD tests and show no reproductive toxicity in the Reproscreen test and the repeated-dose toxicity study. The test results receive reliability 1. The target chemical Cyclaprop is a propyl ester while the source chemicals are ethyl and butyl esters. These differences in chain length are not expected to change the reproductive and developmental toxicity potential of the ester bond.

Expert judgment and empirical findings

The reproductive toxicity of these esters is very low as can be seen in the experimental data available, because the NOAELS are > 1000 mg/kg bw (highest dose tested) and no relevant toxicological effects on the reproductive organs.

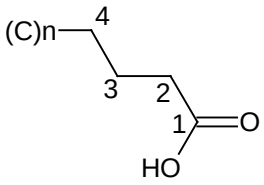
(Q)SAR modelling

The Cycla-esters do not belong to any group of reproductive or developmental toxicants. When using the Blackburn et al (2011) decision tree, the Cycla-ester may all be categorised as 'cyclic compounds with fused polycyclic nucleus', which support the analogue approach. The potential for reproductive and developmental toxicity presented by Blackburn is not seen in the experimental data. In CAESAR the Cycla-esters are also profiled as a similar group. The prediction is that they may be developmental toxicants. This is however not seen in the experimental tests available. The six similar chemicals presented by CAESAR are presented for all Cycla-esters and its alcohol which further support the analogue approach. These six similar chemicals are however very dissimilar to the Cycla-esters.

Common breakdown products or metabolites

In the case of esters, an acid and an alcohol are the metabolites. These metabolites have been assessed during testing of the parent chemicals. No additional experimental information on the Cycla-alcohol is available. This alcohol falls into the Cramer Class I being less reactive compared to Class III. The acids formed are ethyl, propyl and butyl acids and are common acids in the body. Though some types of carboxylic acids are known developmental toxicants, such as valproic acid, the acids of the Cycla-esters are not within this applicability domain. A SAR has been described by DeVito (1996) but the acids of the Cycla-esters do not belong to this group.

Figure B1-4: Structural requirements for high teratogenic potency of carboxylic acids* (DeVito, 1996)



1) A free carboxyl group.
2) Only one hydrogen atom at C-2.
3) An alkyl substituent larger than a methyl at C-2.
4) No double bonds between C-2 and C-3, or C-3 and C-4.

Teratogenic potency based on in vivo data.

*Esters of carboxylic acids that have the requisite structural requirements for teratogenicity are also likely to be teratogenic because esters are often metabolised to the free carboxylic acid.

Similarities in results for toxicological endpoints between the target and the source chemical(s)

As is presented in the data matrix (see B.1.4), the acute oral and dermal toxicity data show limited acute toxicity for both the source and the target chemicals. The negative genotoxicity profile is also similar

between the source and the target chemicals as they are all negative in the Ames test. Both source and target chemical show low systemic toxicity. In the 28-day repeated-dose toxicity study of the source chemical, no reproductive toxic effects were seen at > 1000 mg/kg bw (Cyclobutanate) and the Reproscreen test of Cyclacet. These results show that there is agreement across all toxicity endpoints including the reproductive and developmental toxicity endpoints.

Uncertainty of the prediction

In view of the close resemblance between Cyclacet and Cyclaprop (the latter one having only one methyl group extra), the Reproscreen results of Cyclacet can also be used for Cyclaprop. Also, the results for the other endpoints indicate similarly limited toxicity profiles across the Cycla-esters. The difference in the alkyl chain is not expected to have an impact on the reproductive and developmental toxicity potential. All these Cycla-esters are expected to metabolise into the respective Cycla-alcohol and their acids e.g. due to esterase activity. Therefore the reliability of the prediction of the NOAEL for reproductive toxic effects for Cyclaprop is considered to be 1.

5. Data matrix

See B.1.4.

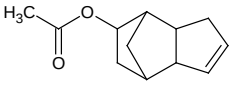
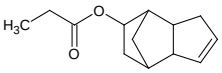
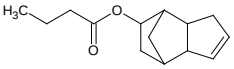
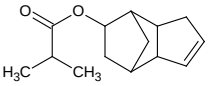
6. Reproductive toxicity prediction for Cyclaprop

For the source chemical Cyclacet, a well-conducted OECD TG test is available (OECD TG 421) (Reliability 1) showing absence of reproductive and developmental dose toxicity at > 1000 mg/kg bw (the highest dose tested) and therefore this absence is also predicted for Cyclaprop. In addition, no reproductive toxic effects were seen in the 28-day study with Cyclobutanate, which has one methyl group extra compared to the target chemical Cyclaprop.

Final conclusion on hazard, C&L, DNEL and risk characterisation

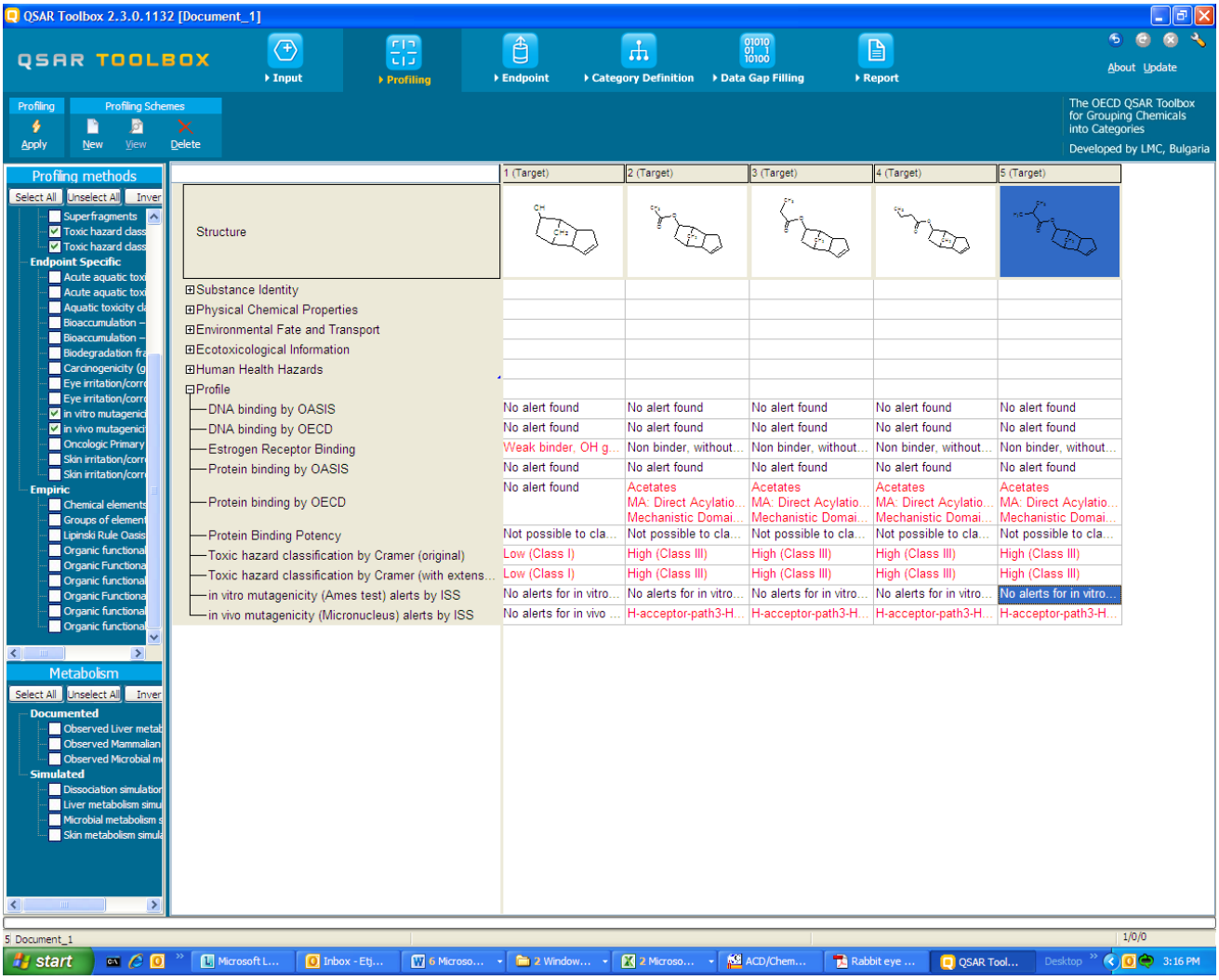
Cyclaprop has no indication for being a reproductive and developmental toxicant and the derived NOAEL is set at 1000 mg/kg bw, based on the Reproscreen test of Cyclacet and the 28-day repeated-dose toxicity study of Cyclobutanate including screening of the reproductive organs. A DNEL for oral, dermal and inhalation route can be based on this information. Classification and labelling are not needed for this endpoint. A risk characterisation will be performed because the Cycla-esters are classified for environmental toxicity. This human health risk characterisation will further support safe use for the reproductive and developmental toxicity endpoints.

B.1.4 Data matrix for Cycla-esters*

Common names	Cyclacet	Cyclaprop	Cyclobutanate	Cyclabute
Chemical structures				
CAS no	2500-83-6 (5-yl)	67634-24-6 (5-yl)	113889-23-9 (5-yl)	67634-20-2 (5-yl)
Physicochemical data				
Molecular weight	192	206	220	220
Physical state	Liquid	Liquid	Liquid	Liquid
Melting point °C	< -20	< -20	< -20	< -20
Boiling point °C	247	264	275	273
Vapour pressure Pa (Episuite)	2.1	0.7	11.2 (EpiSuite: 0.32)	0.6
Water solubility mg/l	186	57	1.5	16
WSK_{ow} mg/l			18	21
Log K_{ow} (meas.)	3.9	4.4	4.48	No data
Log K_{ow} Win	2.85	3.33	3.83	3.76
Log K_{ow} SPARC	3.26	3.74	4.23	4.23
Human health endpoints				
Acute oral toxicity mg/kg bw	3350	Ø 5000	Ø 5000	Ø 2500
Acute inhalation toxicity	No data	No data	No data	No data
Acute dermal toxicity mg/kg bw	>5000	>5000	>2000	Read-across
Skin irritation	Not irritant (OECD TG 404)	Not irritant (OECD TG 404)	Not irritant (OECD TG 404)	Not irritant (OECD TG 404)
Eye irritation	Not irritant (OECD TG 405)	Not irritant (OECD TG 405)	Not irritant (OECD TG 405)	Not irritant (OECD TG 405)
Skin sensitisation	Read-across	Read-across	Not a sensitiser (OECD TG 406)	Not a sensitiser (OECD TG 406)
Genotoxicity – Ames test	Negative (OECD TG 471)	Negative (OECD TG 471)	Negative (OECD TG 471)	Negative (OECD TG 471)
Genotoxicity *in vitro* MLA	Negative (OECD TG 476)	Read-across	Read-across (OECD TG 476)	Read-across
Genotoxicity *in vitro* chromosomal aberration	Read-across	Read-across	Negative (OECD TG 473)	Read-across
Repeated-dose toxicity 28-day mg/kg bw	Read-across	Read-across	NOAEL 1000 (OECD TG 407)	Read-across
Repeated-dose toxicity 90-day	Test is underway (OECD TG 408)			
Reproductive and developmental toxicity mg/kg bw	NOAEL 1000 (OECD TG 421)	Read-across	Read-across	Read-across

* The data presented are based on company-internal studies.

B.1.5 Overview of the OECD (Q)SAR Toolbox profile for the Cycla-esters, and their alcohol



References

Aptula AO, Patlewicz G, Roberts DW. 2005. Skin sensitization: reaction mechanistic applicability domains for structure-activity relationships. Chem Res Toxicol 18:1420-1426.

Aptula AO, Roberts DW. 2006. Mechanistic applicability domains for nonanimal-based prediction of toxicological end points: general principles and application to reactive toxicity. Chem Res Toxicol 19(8):1097-1105.

Blackburn K, Bjerke D, Daston G, Felter S, Mahony C, Naciff J, Robison S, Wu S. 2011. Case studies to test: A framework for using structural, reactivity, metabolic and physicochemical similarity to evaluate the suitability of analogs for SAR-based toxicological assessments. Regul Toxicol Pharmacol 60:120-135.

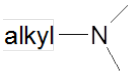
DeVito SC. 1996. General principles for the design of safer chemicals: Toxicological consideration for chemists. In DeVito SC, Garrett RL, eds, Designing safer chemicals, Chapter 2, Vol 640 - ACS Symposium Series. American Chemical Society, Washington, DC, USA, pp 16-59.

ECHA REACH TGD. Technical Guidance Documents for the implementation of REACH. Guidance on information requirements and chemical safety assessment. European Chemicals Agency, Helsinki, Finland. (http://echa.europa.eu/web/guest/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment)

ECHA. 2009. Practical guide 6: How to report read-across and categories. European Chemicals Agency, Helsinki, Finland.

B.2. Alkyldimethylamines (ADA)

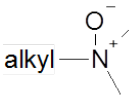
1. Target chemicals



- alkyldimethylamines (ADA), grouped as a category

- alkyl refers to $C_{12\text{-}18}$ chain length, even numbered, no branching

2. Source chemicals



- alkyldimethylamine oxides (ADAO); grouped as a category (OECD HPV)

- oxidation products of ADA

3. Chemistry of ADA

ADA are tertiary amines consisting of two methyl groups and a non-branched long-chain alkyl moiety. The structural variations in the category ADA are twofold:

1. the C-chain-length of the alkyl chain vary from 12 to 18 carbon-atoms
2. the long chain may contain one or more double bonds as in C18:1 (oleyl) or C18:2 (linoleyl).

The origins of long-chain moiety of ADA are natural fatty acids or fatty alcohols which are based on vegetable or animal sources. The fatty acids / alcohols are directly converted by reaction with dimethylamine into the respective substance. The source of the fatty acids / alcohols mostly determines the chain-length distribution and the saturation degree of the products.

Upon uptake in mammals the ADA are degraded by the alpha-C-oxidation. The ultimate metabolites for all ADA differ only by the long-chain fatty acid constituents, corresponding to the given structural variations of the category members. On the other hand, it should be noted that the comparable metabolisms of the long-chain fatty acids as well as their roles in biological systems are well known. Comparable biological activities for the category members can be derived.

4. Use of read-across approach for human health hazard assessment

Insufficient data using ADA as test substances are available to cover all endpoints relevant for the REACH requirements. Especially studies on repeated-dose toxicity and reproduction toxicity are limited. For these endpoints the data sets should be supplemented by the toxicity studies using alkyldimethylamine oxide (ADAO) as test substances.

Such approach is justified by the fact that tertiary amines and corresponding amine oxides are inter-convertible under *in vivo* condition. This view is demonstrated by the available kinetic data on ADAO. Further, identical mode of action can be derived based on all available toxicity data on ADA and ADAO, leading to the comparable toxicity profiles for target and source chemicals.

5. Data matrix*

Endpoints	ADA	ADAO
Repeated-dose oral toxicity	One 28-day study for C12-ADA	studies comprising gavage/feeding/drinking; treatment period up to 2-years; covers all alkyl chain length
Developmental toxicity	no data	studies in rats and rabbits; covers C12 and C12/14 ADAO
Reproduction toxicity	One screening test (OECD TG 421) for C12-ADA	studies comprising gavage/feeding; treatment period up to three-generations; covers all alkyl chain length

* The data presented are based on company-internal studies.

6. Scientific rationale

a) The inter-convertibility of tertiary amines and tertiary amines oxides is one of the basic metabolism principles (Rose and Castagnoli, 1983; Bickel, 1969). Upon oral uptake, the inter-conversion possibly occurs already in the gastro-intestinal tract.

b) *In vivo* kinetic data on ADA are not available. However, the kinetic profile of ADA can be reasonably derived from the results of the kinetic studies on ADAO (Rice, 1977). ADA are expected to be readily bioavailable via oral route and extensively metabolised. The likely degradation pathway comprises ω,β-oxidation, N-oxidation and alpha-C-oxidation. The *in vivo* elimination half-life in rats is likely less than 12h.

c) The kinetic profile of C_{12}ADAO has been investigated in humans, rats and rabbits. Inter-convertibility of C_{12}ADAO and C_{12}ADA could be reasonably derived (Turan and Gibson, 1981).

d) An amine oxidation catalysed by purified rabbit lung monooxygenase could be demonstrated for various sec- and tert-amines including C_8ADA (Poulsen et al, 1986).

e) In mid-term oral toxicity studies both for ADA (28-day) and ADAO (90-day) the gastro-intestinal tract and the associated lymph nodes were identified as target organs. The potency of the observed toxicity was inversely dependent on the alkyl-chain length. These data are serving as bridging data, increasing the robustness of the proposed approach.

f) The outcomes of chronic toxicity, developmental toxicity and 2-generation reproduction toxicity using ADAO as test compound are all in line considering that no systemic effect was found at the dose levels not associated with local damage.

7. Comments on the proposed approach

It is unique that target and source chemicals are metabolites of each other prior to any further degradation. Whichever reaction (N-oxidation or NO-reduction) would be the preceding step, both chemicals share the same degradation pathways, qualitatively and quantitatively, under *in vivo* conditions. Any uncertainty related to different rates or different sites of metabolite formation is not relevant here.

Further, in all available data a local damage was the most distinctive effect, indicating low systemic toxicity associated to the metabolites in relation to the observed local toxicity of parent compounds ADA or ADAO.

The proposed approach was first introduced for the approval of ADA as food contact material. The European Food Safety Authority (EFSA) approved to use the ADAO chronic toxicity data for the safety assessment of ADA (EFSA, 2007).

References

Bickel MH. 1969. The pharmacology and biochemistry of n-oxides. Pharmacological Reviews 21(4):325-355.

EFSA. 2007. Scientific Opinion of the Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request related to a 16th list of substances for food contact materials. European Food Safety Authority. The EFSA Journal 555-563:1-32.

Poulsen LL, Taylor K, Williams DE, Masters BS, DM. 1986. Substrate specificity of the rabbit lung flavin-containing monooxygenase for amines: oxidation products of primary alkylamines. Mol Pharmacol 30(6):680-685.

Rice DP. 1977. The absorption, tissue distribution and excretion of dodecyldimethylamine oxide (DDAO) in selected animal species and the absorption and execretion of DDAO in man. Toxicol Appl Pharmacol 39(3):377-389.

Rose J, Castagnoli N, Jr. 1983. The metabolism of tertiary amines. Med Res Rev 3(1):73-88.

Turan TS, Gibson W.B. 1981. A comparison of the elimination and biotransformation of dodecyldimethylamine oxide (DDAO) by rats, rabbits and man. Xenobiotica 11(7):447-458.

B.3. Montan wax derived chemicals

1. Target chemicals

- Chemicals using montan wax as raw material, chemically processed; grouped as a category

2. Source chemicals

- D-003 (aliphatic acids, from sugar cane wax); pharmaceutics
- Policosanol (aliphatic alcohols, from sugar cane wax); pharmaceutics
- Canauba wax (aliphatic esters, from canauba); food additive

3. Chemistry of target chemicals

- Chemicals using montan wax as raw material, chemically processed; grouped as a category.
- Montax wax refers to esters of aliphatic acids/alcohols, C ≥ 22, even number, no branching.
- Chemical processing refers to extraction/oxidation/distillation. So-called 'very long-chain fatty acid (VLCFA)' and esters of VLCFA/alcohols are produced.
- Di-alcohols such as ethan-di-ol, butan-di-ol could be added as well as CaOH. The final product may contain di-esters of VLCFA as well as Ca-carboxylate of VLCFA.
- Chemicals described as multi-constituent for read-across purpose, whereas each constituent is actually an UVCB due to the chain-length distribution in the raw material.

VLCFA (very-long-chain fatty acid)	$R-COOH$
Esters of VLCFA and alcohol	$R-COO-R'$
Esters of VLCFA and ethan-di-ol	$R-COO-CH_2CH_2-OOC-R'$
Esters of VLCFA and butan-di-ol	$R-COO-CH_2CH(CH_3)CH_2-OOC-R'$
Ca-carboxylate of VLCFA	$Ca(R-COO)_2$

- Extensive analytical work performed. A definitely quantified set of constituents could be assigned to each category member. Chemical elements that deviate from the VLCFA definition were identified, quantified and assigned as impurities.

4. Use of read-across approach for human health hazard assessment

Available toxicity data for target chemicals are insufficient, i.e. do not cover all endpoints relevant for the REACH requirements. Especially studies on repeated-dose toxicity and reproduction including developmental toxicity are limited. For these endpoints, the data sets should be supplemented by the toxicity data on source chemicals. The underlying hypothesis is that there is a functional commonality between target and source chemicals.

5. Data matrix*

Endpoints	Target chemicals	Source chemicals
Repeated-dose oral toxicity	One combined repeated-toxicity and reproduction screening test (OECD TG 422) using a category member with highest VLCFA content	Studies comprising gavage/feeding; treatment period up to 2-years; available for all source chemicals
Developmental toxicity	One developmental toxicity study (OECD TG 414) using a category member with highest ester content	Studies in rats and rabbits; available for all source chemicals
Reproduction toxicity	One screening test (OECD TG 421) using a category member with a highest ester content	Studies comprising gavage/feeding; treatment period up to multi-generations; available for all source chemicals

* The data presented are based on company-internal studies.

6. Scientific rationale

a. The target chemicals are composed of acids, alcohols or esters of VLCFA. VLCFA is bioavailable and extensive metabolism occurs after uptake (Drover et al, 2008; Menéndez et al, 2005). Esters of VLCFA undergo enzymatic hydrolysis prior to oral resorption (Hargrove et al, 2004). VLCFA and alcohols are formed as metabolites.
b. Chemical descriptions of source chemicals are identical to those of constituents of target chemicals and/or their hydrolysis metabolites.
c. Low toxicity for all endpoints required by REACH is demonstrated by the extensive data set on the source chemicals. The available data on the target chemicals are exhibiting practically no effect.
d. Additional support for low toxicity of target and source chemicals is indicated by the biological roles of VLCFA[3] (Van Duyn et al, 1984). Humans are exposed to the endogenously produced VLCFA as well as via food intake. The biological role of VLCFA and adverse effects arising from the impaired metabolism are well understood. The VCLFA exposure through chemical use is negligible in comparison to estimated turn-over of VCLFA of other origins.

7. Comments on the proposed approach

The chemical description of the target and source chemicals was the key argument for the use of the proposed read-across approach. The target chemicals were characterised as multi-constituent substances, so that the functional commonality between each constituent and selected source chemical could be established. Absence of any toxicity across all endpoints, which can be explained by mechanistic considerations, increases the robustness of the proposed approach.

[3] http://en.wikipedia.org/wiki/Adrenoleukodystrophy

References

Drover VA, Nguyen DV, Bastie CC, Darlington YF, Abumrad NA, Jeffrey E. Pessin JE, London E, Sahoo D, Phillips MC. 2008. CD36 mediates both cellular uptake of very long chain fatty acids and their intestinal absorption in mice. J Biol Chem 283(19):13108-13115.

Hargrove JL, Greenspan P, Hartle DK. 2004. Nutritional significance and metabolism of very long chain fatty alcohols and acids from dietary waxes. Exp Biol Med (Maywood) 229(3):215-226.

Menéndez R, Marrero D, Más R, Fernández I, González L, González RM. 2005. *In vitro* and *in vivo* study of octacosanol metabolism. Arch Med Res 36(2):113-119.

Van Duyn MA, Moser AE, Brown FR 3rd, Sacktor N, Liu A, Moser HW. 1984. The design of a diet restricted in saturated very long-chain fatty acids: therapeutic application in adrenoleukodystrophy. Am J Clin Nutr 40(2):277-284.

B.4. Glycol ethers E series category

1. Category hypothesis

This category covers E series glycol ethers that are produced by the reaction of ethylene oxide (EO) with primary and secondary alcohols in the range C1-C6 (methanol to hexanol). Not all of the members are commercially important (e.g. isobutanol and pentanol derived glycol ethers are not produced and therefore there is no data on them). Category members can have one or more EO 'monomer' units in them, although the maximum number for discrete molecules is four. Molecules containing a higher number of EO units do exist but these are normally present as impurities only or as minor components of the higher molecular weight substance streams from production processes. The generic structure is therefore:

$$C_nH2_{n+1}.(OCH_2CH_2O)_m.H$$

where n is 1 to 6 and m is typically 1 to 4

The category is characterised by the presence of one or more ether groups and a single primary hydroxyl group. The hypothesis is that members of this category undergo similar metabolic pathways, with the main metabolite derived through oxidation of the hydroxyl function to a carboxylate group and that, for systemic endpoints, the acid metabolite determines the toxicity of the glycol ether rather than the parent glycol ether itself. A number of endpoints are data rich and many contain multiple values enabling clear patterns to emerge of how physicochemical properties change and toxicity increases or decrease across the homologous series. This enables interpolation and, with care, extrapolation to be used to predict the toxicity for members of the category where data is not available. These trends are shown graphically in the list of endpoints covered in section 3.

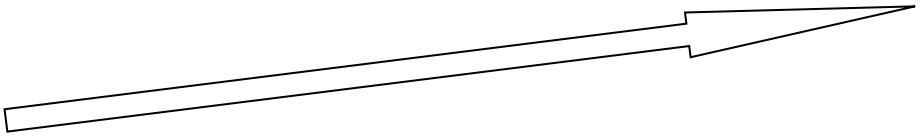
2. Applicability domain of the category

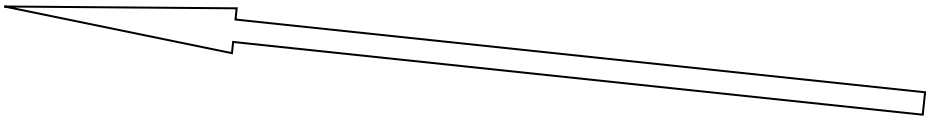
The category applies to all linear E series glycol ethers and the branched isopropyl glycol ether. Scope is limited to those made using alcohols up to C6 as there is no data beyond this and no need to extrapolate the category beyond this point. The category applies to glycol ethers containing up to four linked ethylene oxide units. The category does not cover glymes (molecules containing two alcohol units and characterised by no hydroxyl functionality).

3. List of endpoints covered and trends analysis for each endpoint

All endpoints can be considered in scope. The apparent trends for each are described in this section.

Physicochemical endpoints

PROPERTY	INCREASING PARTITION COEFFICIENT DECREASING DENSITY, WATER SOLUBILITY					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono						
Di						
Tri						
Tetra						

PROPERTY	INCREASING SURFACE TENSION, AUTOIGNITION TEMPERATURE, FLASH POINT DECREASING MELTING POINT, BOILING POINT, VISCOSITY					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono						
Di						
Tri						
Tetra						

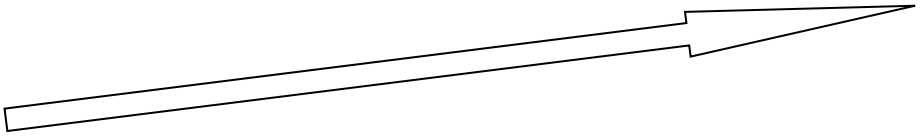
PROPERTY	DISSOCIATION CONSTANT					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono	All category members have a predicted dissociation constant of 14.9±0.1					
Di						
Tri						
Tetra						

Trends observed: Most of the physicochemical endpoints are available for the more important members of the category. All of the endpoints show clear and expected trends across and down the category, including for the more important endpoints such as the vapour pressure and partition coefficient. Some endpoints show almost constant values across the category. Water solubility shows complete water miscibility until the break point where the hydrophobicity of the category members increases to the point where solubility becomes finite. Where there are data gaps, these can be confidently filled by both interpolation between and extrapolation from the data that does exist.

Environmental toxicity endpoints

Ecotoxicity

PROPERTY	INCREASING TOXICITY TO FISH, INVERTEBRATES, ALGAE, MICRO-ORGANISMS					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono						
Di						
Tri						
Tetra						



Trends observed: This class of chemicals does not show specific toxic mechanisms. Toxicity is via non-specific narcosis and is directly proportional to the partition coefficient of the substance. This is supported by the fact that toxicities across all tropic levels are within a similar order of magnitude. As such, there are clear trends across the group, with toxicity increasing with increasing alcohol chain length and decreasing with the increasing number of EO moieties in the molecule. Interpolation between adjacent members of the category can be considered a reliable and justified approach. Similarly, extrapolation in a 'less toxic direction', i.e. from the top right of the category map down and/or to the left can be considered justifiable.

Biodegradability

PROPERTY	BIODEGRADATION					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono						
Di	All category members based on linear alcohols are readily biodegradable.					
Tri	The limited evidence from the isopropyl series indicates that these are at least inherently biodegradable.					
Tetra						

Trends observed: Across the category, there is sufficient data to conclude that all E series glycol ethers derived from primary linear alcohols will be readily biodegradable. The limited evidence available suggests that those derived from branched members may only be inherently biodegradable. Most members of the category have biodegradation data available. Again, for those that do not have the clear trend amongst the category implies that these conclusions can be reliably extended to those category members with missing information.

Mammalian toxicity endpoints

Acute toxicity

PROPERTY	ACUTE TOXICITY , ALL ROUTES, INCREASING TOXICITY					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono						
Di						
Tri						
Tetra						

■ Classified as harmful by at least one route of exposure. The monopentyl is not formally classified as it is not marketed commercially so this is a projection.

Trends observed: The relatively imprecise nature of LD_{50} values coupled with the fact that not all toxicity values are available in the same species means that caution needs to be exercised in concluding trends for this endpoint. This is mainly an issue when comparing between routes; for the oral and inhalation routes, a good data set is available in rats and for the dermal route there is a good data set in rabbits. There does appear to be a variation in sensitivity to different species. However, for all routes of exposure, there is a clear trend towards reducing toxicity vertically down a homologous series; increasing the number of EO moieties clearly reduces toxicity. There is no clear trend across the series produced with different alcohols and in fact the toxicity does not seem to change much between members of the category based on different alcohols (for a given number of EO moieties). Other data from the studies beyond the headline LD_{50} also indicate that differences in the alcohol chain can cause different critical effects, which suggests further caution in extrapolating horizontally. That being said, all of the mono series are classified as harmful for acute toxicity by the oral and dermal routes and also for the inhalation route where the vapour pressure is high enough (>~1hPa). The exception to this is the propyl series, neither of which is classified for the oral route and n-propyl is only classified for the dermal route. This anomaly may be caused by relative species sensitivity to critical effects such as haemolysis and reflect the available species data for the routes of exposure for this endpoint or may even be a consequence of the variability of this assay, as already mentioned. However, it does reinforce the observation that read-across between alkyl series must be done with caution. In conclusion, for acute toxicity, extrapolation and interpolation within a single alcohol homologous series seems entirely justified and the results of such an approach can be considered reliable and robust. Extrapolation horizontally across a group is not likely to be reliable although interpolation can be justified.

Skin irritation

PROPERTY	INCREASING SKIN IRRITATION					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono					*	
Di						
Tri						
Tetra						

* Predicted classification based on trends.
Classified as irritating. Shaded cell indicates that isopropyl is classified but n-propyl is not.
Classified as causing burns.

Trends observed: Data are available for most of the important category members and shows a clear trend of increasing skin irritancy with increasing chain length of the alcohol used and decreasing number of EO moieties present. As such, interpolation between adjacent members of the category can be considered a reliable and justified approach. Similarly, extrapolation in a 'less toxic direction', i.e. from the top right of the category map down and/or to the left can be considered justifiable and reliable.

Eye irritation

PROPERTY	INCREASING EYE IRRITATION					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono					*	
Di					*	
Tri						
Tetra						

* Predicted classification based on trends.
Classified as irritating.
Classified as damaging.

Trends observed: Data are available for most of the important category members and shows a clear trend of increasing eye irritancy with increasing chain length of the alcohol used and increasing number of EO moieties present. (As a point of note, this seems to be the only endpoint where toxicity increases with increasing molecular weight within a given homologous series, although the detailed data from the available studies indicates that the alcohol used is the more important determinant rather than the number of EO units.) As such, interpolation between adjacent members of the category can be considered a reliable and justified approach. Similarly, extrapolation in a 'less toxic direction', i.e. from the bottom right of the category map up and/or to the left can be considered justifiable and reliable.

Repeated-dose toxicity

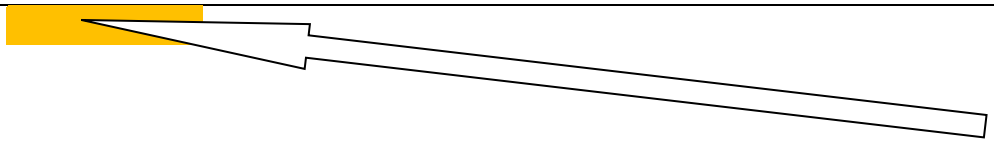
Trends observed: There are arguably two distinct substance-specific toxicological findings from repeated-dose studies on E series glycol ethers with two different trends for the two different effects. These two key

effects vary consistently across the series and as with other endpoints, toxicity results from the effects of the acid metabolite rather than the parent glycol ether:

- The low molecular weight E series glycol ethers exert detrimental effects on the haematopoietic system in the bone marrow (blood cell formation) and cause a deficiency of all cell elements (red and white) of haematopoiesis (generalised pancytopenia) in test animals and humans. Broader adverse effects on the lymphoid organs and tissues are also seen, including marked reductions in thymus weight, specific antibody reduction and increased NK cell cytotoxicity. Whilst some adverse changes have been reported for both DEGME and EGEE, only glycol ethers that can be metabolised to methoxyacetic acid are considered to be specifically immunotoxic and therefore effects are only seen at significant levels with methoxyethanol.
- In contrast, monoalkyl glycol ethers around the C4 range exert detrimental effects only to mature red blood cells in the peripheral blood; the erythrocyte membranes show increased osmotic fragility and are subject to intravascular lysis. The haemolytic effect, which is caused by the acid metabolite rather than the parent glycol ether, does not affect white blood cells and is species-specific (mice, rats and rabbits show haemolysis; guinea pigs are more resistant; it does not occur in humans, even in sub-populations that might be considered more vulnerable). The effect is not seen with di- and trialkyl glycol ethers. It is of note that this effect can also be seen following a single (acute) exposure and that a degree of resistance can build up as it is particularly older red blood cells that are most vulnerable. These effects do seem to be confined to the mono member of the category; no haemolytic activity is reported for DEGBE.

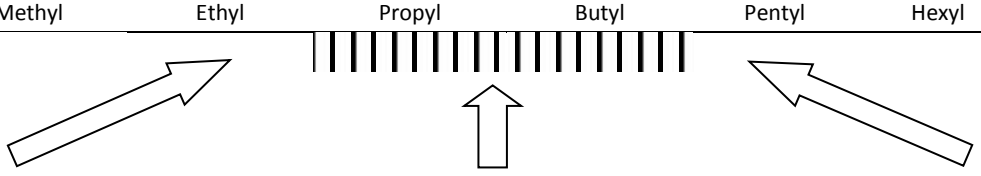
The trends for these two effects are shown in the figures below:

PROPERTY	**REPEATED-DOSE TOXICITY, INCREASING IMMUNOTOXIC EFFECTS**					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono						
Di						
Tri						
Tetra						



Classified for specific target organ toxicity (immunotoxicity).

PROPERTY	**REPEATED-DOSE TOXICITY, INCREASING HAEMOLYTIC EFFECTS**					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono						
Di						
Tri						
Tetra						



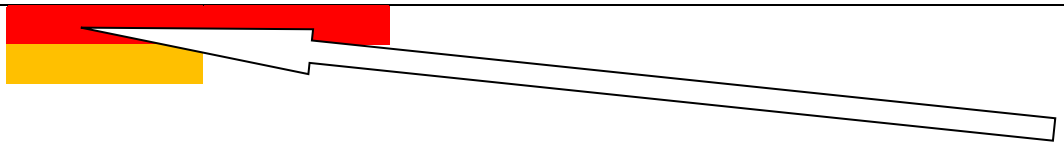
Toxic effect seen, not classifiable.

For this endpoint, it might be useful to consider the introduction of sub-categories. For immunotoxic effects it is probably simpler to consider excluding methoxyethanol from the category as a whole as this is the only glycol ether that shows this effect. However, since there is some evidence for the effects with glycol ethers adjacent to EGME, even if these are not at levels that cause concern, it seems more appropriate to consider the category still as a whole. For the haemolytic effects, since these are not relevant to humans and no classification therefore results from them, there seems to be little benefit from considering a sub-category approach.

As for acute toxicity, across all of the routes of exposure, the amount of available data is quite comprehensive, at least for the commercially produced glycol ethers. The ability to test by the inhalation route is limited by vapour pressure, with a limiting vapour pressure of ~1hPa determining the cross-over point where vapour pressure limits the ability to manifest adverse effects on exposure. For the oral route, most of the data is available in rats, whilst for the dermal route the majority of the available data is in rabbits. Whilst there is no single trend, due to there being more than one toxic effect, it is clear that there is a marked decrease in toxicity with increasing number of EO moieties in the molecule.

Toxicity to reproduction

PROPERTY	**TOXICITY TO REPRODUCTION INCLUDING DEVELOPMENTAL TOXICITY INCREASING TOXICITY**					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono						
Di						
Tri						
Tetra						



Category 2 for developmental toxicity.
Category 1B for reproductive and developmental toxicity.
Note that those not classified do not show any adverse reproductive effects in available studies.

Trends observed: The relationship between structure and reproductive or developmental toxicity of the glycol ethers follows the broad principles established for other toxicity endpoints. Oxidation to the respective alkoxyacetic acid is a prerequisite for the expression of adverse reproductive effects, with the acid metabolite mediating toxicity. There is clear data to show that all reprotoxicity endpoints, including developmental toxicity and fertility decrease markedly as the lengths of the alkyl and alkoxy chains increase. For reproductive toxicity, the most sensitive effect is on the male testes, which manifest as testicular atrophy and arrestment of spermatogenisis. From the available data, the most toxic member of the category is methoxyethanol for all reproductive endpoints. Ethylene glycol ethers with alkyl chains of three or more carbon atoms do not express reproductive toxicity. The trend towards manifest reproductive toxicity in the short-chain glycol ethers is at least in part related to the trend for slower elimination of these metabolites with decreasing alkyl chain length and decreasing numbers of EO units in the molecule. It is also possible that the marginal adverse effects seen with DEGME may be due to a minor metabolic pathway leading to small amounts of systemic methoxyethanol and hence methoxyacetic acid. For those glycol ethers where developmental data is available in multiple species, there is no evidence for any species-specific adverse effects, suggesting that data from a single species should be sufficient to characterise developmental effects.

The ideal situation in terms of read-across and extrapolation would be to have data on all of the lower molecular weight members until a 'boundary' was evident, delineating the point at which toxicity decreased to a negligible level, and at which point it would be valid to extrapolate in any direction to the right or downwards on the category map to fill an empty data gap. The pattern of decline of toxicity across the series suggests that, it should be possible to extrapolate upwards and to the right as well as downwards and to the left and assume that toxicity is similar (rather than reduced as is the case when extrapolating downwards and to the right). This is inherently less conservative and should only be used as part of a weight of evidence approach when there is data for the target read-across glycol ether but it is not of sufficient quality or of the right study type to fill the data gap on its own. The conclusion is therefore:

- For substances with no data: Extrapolation from a substance with data that is to the left and upwards of the target glycol ether is valid and robust.
- For substances with partial but incomplete data for the endpoint: Extrapolation from a substance with data that is to the left and below or to the right and above is acceptable as part of a weight of evidence approach.

For this endpoint, a sub-category approach is an option and glycol ethers are cited as an example of this approach in the ECHA guidance (ECHA REACH TGD). Following this approach, the three glycol ethers showing reprotoxicity would be considered in one category whilst the remaining non-toxic ones would be in a separate sub-category. However, in the context of the approach used in this document, there is no advantage to do this since there is sufficient data available to support the trends for all endpoints, including reprotoxicity, and these data clearly show where effects are significant or not across the category when treated as a whole.

Mutagenicity and skin sensitisation

PROPERTY	**SKIN SENSITISATION, MUTAGENICITY**					
Number of EO units	**Alcohol chain length**					
	Methyl	**Ethyl**	**Propyl**	**Butyl**	**Pentyl**	**Hexyl**
Mono						
Di	No member of category is a skin sensitiser					
Tri	No member of the category has mutagenic properties					
Tetra						

Trends observed: For both of these endpoints, there is clear evidence across the whole category that the E series glycol ethers do not show significant mutagenic or skin sensitisation properties. Extrapolation or interpolation of this same conclusion to members of the category without data seems scientifically justified.

4. Category members

MONOETHYLENE GLYCOL ETHERS				
Abbreviation	**Chemical and other names**	**Smiles code**	**CAS no**	**Mol wt (g/mol)**
EGME	2-methoxyethanol Ethylene glycol methyl ether	COCCO	109-86-4	76.1
EGEE	2-ethoxyethanol Ethylene glycol ethyl ether	CCOCCO	110-80-5	90.1
EGnPE	2-(propyloxy)ethanol Ethylene glycol n-propyl ether	CCCOCCO	2807-30-9	104.2
EGiPE	2-isopropoxyethanol Ethylene glycol isopropyl ether	CC(C)OCCO	109-59-1	104.2
EGBE	2-butoxyethanol Ethylene glycol butyl ether	CCCCOCCO	111-76-2	118.2
EGPeE	2-pentoxyethanol Ethylene glycol pentyl ether	CCCCCOCCO	6196-58-3	132.2
EGHE	2-hexyloxyethanol Ethylene glycol hexyl ether	CCCCCCOCCO	112-25-4	146.2

DIETHYLENE GLYCOL ETHERS				
Abbreviation	**Chemical and other names**	**Smiles code**	**CAS no**	**Mol wt (g/mol)**
DEGME	2-(2-methoxyethoxy)ethanol Diethylene glycol methyl ether	COCCOCCO	111-77-3	120.2
DEGEE	2-(2-ethoxyethoxy)ethanol Diethylene glycol ethyl ether	CCOCCOCCO	111-90-0	134.2
DEGnPE	2-(2-propoxyethoxy)ethanol Diethylene glycol butyl ether	CCCOCCOCCO	6881-94-3	162.2
DEGBE	2-(2-butoxyethoxy)ethanol Diethylene glycol butyl ether	CCCCOCCOCCO	112-34-5	162.2
DEGHE	2-(2-hexyloxyethoxy)ethanol Diethylene glycol hexyl ether	CCCCCCOCCOCCO	112-59-4	190.3

TRIETHYLENE GLYCOL ETHERS				
Abbreviation	**Chemical and other names**	**Smiles code**	**CAS no**	**Mol wt (g/mol)**
TEGME	2-(2-(2-methoxyethoxy)ethoxy)-ethanol Triethylene glycol methyl ether	COCCOCCOCCO	112-35-6	164.2
TEGEE	2-(2-(2-ethoxyethoxy)ethoxy)-ethanol Triethylene glycol ethyl ether	CCOCCOCCOCCO	112-50-5	178.2
TEGBE	2-butoxyethanol Triethylene glycol butyl ether	CCCCOCCOCCOCCO	143-22-6	206.3

TETRAETHYLENE GLYCOL ETHERS				
Abbreviation	**Chemical and other names**	**Smiles code**	**CAS no**	**Mol wt (g/mol)**
TetraEGME	2-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)ethanol Tetraethylene glycol methyl ether	COCCOCCOCCOCCO	23783-42-8	208.3
TetraEGBE	2-(2-(2-(2-butoxyethoxy)ethoxy)ethoxy)ethanol Tetraethylene glycol butyl ether	CCCCOCCOCCOCCOCCO	1559-34-8	250.3

5. Purity/impurities

The monoethylene and diethylene glycol ethers are invariably >99% pure, with more typical actual purities around 99.5%. Impurities can include both lower and higher members of the alcohol homologous series (with fewer or more EO moieties), the parent alcohol used or members of the ethylene glycols through self-reactions, including di- and trimerisation etc. of the ethylene oxide. Impurities of potential concern are controlled to very low levels.

The exceptions in terms of purity are the higher molecular weight members of the category. Whilst the triethylene glycol alkyl ethers are often supplied as 'pure' they can contain 5-10% by weight of the tetra ethylene glycol alkyl ethers, and sometimes smaller amounts of the lower members of the family as well. The tetras are not supplied in pure form. However, 'heavy' glycol ether streams are marketed and these can contain mixtures of predominantly the tri- and tetra-members but also significant amounts of penta, hexa, hepta molecules (containing 5, 6 and 7 EO units respectively). These 'heavy' streams will not contain any significant amounts of ethylene glycols.

Note that no mixtures across alcohol series are produced and the presence of E series glycol ethers of with different alcohol parents would not normally occur as impurities except through contamination in the supply chain or in storage.

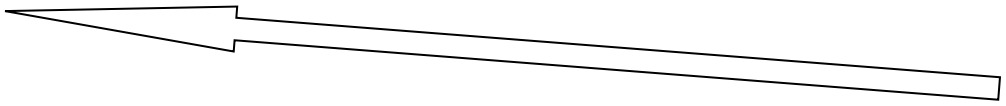
6. Category justification

The E series glycol ethers show clear trends in water misciblity, partitioning behaviour and vapour pressure. Water solubility decreases and the partition coefficient increases with increasing alkyl chain length; all those from butyls and below are fully water miscible and have quite low log K_{ow} values. All members of the category have moderate to low vapour pressure. There toxicity in environmental species is by non-specific narcosis, which means that toxicity behaviour across a set of representative members of the category is highly predictable. For some endpoints, data exists for most members of the category. From the data available, it is possible to see that there are clear patterns for all of the endpoints in terms of the way toxicity changes across the category. From these trends it is possible to interpolate between two adjacent members of a category for which data exists (either using the worse-case result as the read-across value) or it is possible to extrapolate in the direction of decreasing toxicity, using the result from the more toxic member of the category as a conservative read-across value for the less toxic member for which no data is available.

For environmental toxicity, the ECOSAR (Q)SAR supports the trend shown. However, the measured data suggests that the diethylene glycol members may be the most toxic members of each homologous series. This needs to be borne in mind during any interpolation or extrapolation.

The E series category of glycol ethers share common paths of adsorption, distribution, metabolism and elimination. The remainder of this section is derived from the ECETOC technical report 95 on "The toxicology of glycol ethers and its relevance to man" (ECETOC, 2005).

Glycol ethers are readily absorbed following oral administration or inhalation. Dermal absorption is also an important exposure route; penetration rates in human epidermis *in vitro* have shown a rank order for liquid contact: EGME > EGEE > EGBE > DEGME > DEGEE > DEGBE. This shows a clear trend of increased dermal penetration with decreasing molecular size, as would be expected. This is shown in the following figure:

PROPERTY	**INCREASING DERMAL PENETRATION** **POSSIBLE DECREASE IN RATE OF ELIMINATION OF ALKOXYACETIC ACID METABOLITE**					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
Mono						
Di						
Tri						
Tetra						

Once absorbed, E series glycol ethers are readily distributed throughout the body; no substantial bioaccumulation of the parent compound has been observed. However, the alkoxyacetic acid metabolites of EGME and EGEE have shown evidence of accumulation in animals and humans whilst in contrast, the metabolite of EGBE shows no evidence of significant accumulation This suggests that elimination of this metabolite, which is invariably the proximate toxicant (see below) decreases with reducing alkyl chain length and number of EO units.

E series glycol ethers can follow two main oxidative pathways of metabolism, either via ADH or the microsomal CYP mixed function oxidase (MFO) (O-demethylation or O-dealkylation). The first and dominant pathway gives rise to the formation and excretion of alkoxyacetic acids. The second mainly leads to the production and exhalation of carbon dioxide (CO_2) via ethylene glycol (MEG) , which enter intermediary metabolism via the tricarboxylic acid (TCA) cycle. In addition to these two pathways, conjugation with sulphate, glucuronic acid or glycine has also been reported.

All monoethylene glycol ethers bearing a primary OH-group (alkoxyethanols) are primary alcohols that are oxidised via ADH and aldehyde dehydrogenase (ALDH) to their corresponding alkoxyacetic acids. This is the dominant route of metabolism for the mono series. The toxicity profiles of these acids are very similar to their parent compounds. Investigations at the *in vivo* and the *in vitro* level have shown that nearly all effects of this group of glycol ethers are mediated by these metabolites. The bioavailability and toxicity of these metabolites depends largely on the dose but also the metabolic rate and species (with toxicity increasing

with reduced rates of excretion/elimination). The likely trend in the rate of metabolism across the category is shown in the diagram above.

Diethylene (and tri and tetra) glycol ethers may undergo a low level formation of ether cleavage and formation of alkoxyacetic acids. These dialkoxyacetic acids seem to show far lower levels of toxicity than their mono equivalents. The tri- and tetra-equivalents are predicted to be of even lower toxicity.

An important point of note is that for all of the important toxic effects of glycol ethers (those requiring systemic delivery to the site of effect) the proximate toxicant is the alkoxyacetic metabolite and not the parent glycol ether itself.

7. Data matrix for glycol ether E series chemical category

The available data should be considered in the context of which of the E series glycol ethers are commercially important. These are shown below. Blocked out cells are no longer considered to be commercially important members of the category. In addition, the table also shows the dates when REACH registration dossiers have been or will be submitted for each substance.

	COMMERCIALLY IMPORTANT MEMBERS OF THE CATEGORY						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	2010		2010	2010	2010		2010
Di	2010	2010			2010		2013
Tri	2010	2010			2010		
Tetra*	2010*				2010*		

* Not in pure form but as a mixture of tri, tetra and heavier members of the series. Dossiers have been submitted under REACH as UVCB covering process streams that are primarily a mixture of the tri and tetra components. In the case of butyls, more than one CAS number has been registered.

Note that in the tables below, which show where actual data is available for each substance for each endpoint, orange and red highlighting indicate an endpoint where the data indicates classification is required under regulation 1272/2008 (red is a more severe classification than orange). For data sources, see References.

Physicochemical properties

Property	Melting point (C)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	-85	-70	<-20	-60	-75		-50
Di	-84	-54			-66		-35
Tri	-44	-19			-35		
Tetra							

Property	Boiling point (C)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	124	136	147	145	171		208
Di	193	196	215		230		258
Tri	250	256			278		
Tetra							

Property	Density (kg/m^3)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	960	926	911	903	900		888
Di	1020	989	963		955		937
Tri	1049	1020			989		
Tetra							

Property	Vapour pressure (Pa)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	1260	766	643	378	117		10
Di	24	17	2.7		2.9		<0.1
Tri	1.0	~1.0			0.33		
Tetra							

Property	**Partition coefficient**						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	0.77		0.673	0.43	0.81		1.97
Di	-0.47	-0.54			1.0		1.27
Tri	-1.12				0.51		
Tetra							

Property	**Water solubility (g/l)**						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	miscible	miscible	miscible	miscible	miscible		9.5
Di	miscible	miscible			miscible		18
Tri	miscible	miscible			miscible		
Tetra							

Property	**Surface tension (mN/m)**						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	70@1g/l		71		65@2g/l		
Di	65@25g/l						
Tri	65@25g/l				61@1g/l		
Tetra							

Property	**Flash point (C)**						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	42	49	51	46	67		91
Di	91	91	93		115		133
Tri	110				131		
Tetra							

Property	Autoignition temperature (C)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	285	235	256	240	230		225
Di	215	204	204		210		
Tri	210				202		
Tetra							

Property	Dissociation constant (by QSAR)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	14.8	15.0	15.0	15.0	15.0		15.0
Di	14.9	14.9			14.8		14.9
Tri	14.9	14.9			14.9		
Tetra							

Property	Viscosity (mPa.s)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	1.7		2.97	2.6	3.3		4.4
Di	3.9	4.4			6.0		
Tri	7.3	7.9			9.2		
Tetra							

Notes: Values for n-propyl, monoethyl and dihexyl obtained from Patty's Industrial Hygiene, 5th ed, Glycols and glycol ethers. Dissociation constants calculated. All other data from ECHAChem.

Environmental Fate

Property	Photodegradation (by QSAR) – Half life (HRS)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	10.7	7.4	5.9	5.5	5.5-16*		14.6
Di	4.9	4.1	3.6	3.4	3.4		3.2
Tri	3.2	2.8	2.6	2.5	2.5		
Tetra							

* Also measured data of 26% degradation after 6 hours in smog chamber.

Property	Aerobic biodegradation screening results						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	RB		RB	IB	RB		RB
Di	RB	RB			RB		RB
Tri	RB	RB			RB		
Tetra							

RB = Readily biodegradable. IB = Inherently biodegradable.

Environmental Toxicity

Acute toxicity (freshwater)

Property	Fish LC_{50} (mg/l)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	10000	10000	>5000	>100*	1474		140
Di	5741	6010			1300		13000
Tri	10000	10000			2400		
Tetra							

* Limit test, QSAR prediction is 5000.

Property	Invertebrates EC_{50} (mg/l)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	9400	7700	>5000*	>970*	690		145
Di	1192	1982			>1000		433
Tri	10000	10000			2210		
Tetra							

* Limit tests.

Property	Algae EC_{50} (mg/l)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	>10000	>1000	>100	>1000	911		147
Di	>1000	>100			>100		>100
Tri	4975				>620		
Tetra							

Property	Micro-organisms EC_{50} (mg/l)					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
			n- iso			
Mono	>10000		>1000			750*
Di	>10000	>5000				
Tri		36000				
Tetra						

* EC_{20}

Property	Micro-organisms NOEC (mg/l)					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
			n- iso			
Mono	>1000	1730	4600	463		
Di	10000			255		>1000
Tri		7500		1995		
Tetra						

Chronic toxicity

Property	Fish NOEC (mg/l)					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
			n- iso			
Mono				>100		
Di						
Tri						
Tetra						

Property	Invertebrates NOEC (mg/l)					
Number of EO units	Alcohol chain length					
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
			n- iso			
Mono	>500		98	100		
Di		7.4*				20
Tri						
Tetra						

* Value from a single study is surprisingly low and out of line with acute data, data from other glycol ethers and QSARs.

Property	Algae NOEC (mg/l)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	>1000			>1000	88		23
Di		>100					>100
Tri	1000				63		
Tetra							

Mammalian Toxicity

Abbreviations: Gp=guinea pig, Rb=Rabbit, Rt=rat, Ms=Mouse. Figures lower than the preferred species (usually rat) are shown. Figures above those for preferred species are not shown.

Acute toxicity

Property	Oral LD_{50} (g/kg) –RAT unless otherwise indicated						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	2.2-3.9 Gp=0.95	2.1-6.6 Gp=1.4	3.1 Ms=1.8	>2	1.3 Rb<0.65	*	0.7-1.5
Di	7-12 Gp=4.2	6.0 Gp=3-5			3.3 Ms=2.4 Rb=2.2 Gp=2		3.5-4.6
Tri	>10.5	10.6			5.2		
Tetra							

See section 1.3 for explanation of anomaly for propyl series
* = Predicted classification.

Property	Inhalation LC_{50} (mg/l) – RAT unless otherwise indicated						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	16	16	>9	15	2.2* Gp>SVP		>SVP
Di	>SVP	>SVP			>SVP		>SVP
Tri	Waived	Waived			waived		
Tetra							

> SVP = greater than saturated vapour pressure. *=close to SVP. Many tests do not shown an LC_{50} at SVP.
See section 1.3 for explanation of anomaly for n-propyl.

Property	Dermal LD_{50} (g/kg)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	Rb=1.34	Rb=3.3	Rb=1.3†	Rb=1.4†	1-2† Rb~0.5, Gp>2	*	0.8
Di	Rb=9.3 Gp=5-8	Rb=9.1 Gp=5.9			>2 Rb=2.8		Rb=1.4-2.2
Tri	Rt, Rb=7.45*				Rb=3.5		
Tetra							

Note that species used for oral and dermal routes not always the same so comparison may not be valid. imilarly comparison within the dermal values can similarly be confounded. * = Predicted classification. †Haemolysis seen

Irritation and skin sensitisation

Property	Skin irritation						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	no	no	NO	Yes H315	Yes H315	*	Yes H314
Di	no	no			No		Yes H315*
Tri	no				No		
Tetra							

* Predicted to be classified as a skin irritant. ** Based on available data, likely to be a skin irritant.

Property	Eye irritation						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	no	no	Yes. H319	Yes. H319	Yes. H319	*	Yes. H318
Di	no	no			Yes. H319		Yes H318
Tri	no				Yes. H318		
Tetra							

* Predicted to be classified as an eye irritant.

Property	Skin sensitisation						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	no		No		No		No
Di	no	no			No		No
Tri							
Tetra							

Repeated-dose toxicity

Property	Sub-chronic ORAL NOAEL (mg/kg/day) – RAT unless otherwise indicated						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	15.6	93	<195†	<30*†	<69†		
Di	900 (6 wk)	Dg=1000			250†		300
Tri	400						
Tetra							

* = Sub-acute study. †critical effect is haemolysis that humans are not sensitive to.

Property	Sub-chronic inhalation NOAEC (mg/m^3)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	95 Rb<95	Rt, Rb=375	433†	6 months 108†	<152†		250
Di	>SVP	>SVP			94*		
Tri	Waived	Waived			Waived		
Tetra							

† Critical effect is haemolysis that humans are not sensitive to. * = Aerosol. No effects from vapour up to SVP.

Property	Sub-chronic DERMAL NOAEL (mg/kg/day)						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	100				Rb=150†		Rb=222‡
Di	Gp=40§	>1000 (28 day)			2000		Rb>1000‡*
Tri	4000				Rb>1000*		
Tetra							

† Critical effect is haemolysis that humans are not sensitive to. * Sub-acute study only. ‡ Systemic effects. Site of contact irritation seen at lower doses (substance is a severe skin irritant). § Questionable reliability of NOAEL.

Genotoxicity

	Overall evidence						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	NO		NO	NO	NO		NO
Di	NO	NO			NO		NO
Tri	NO				NO		
Tetra							

	Available test data						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	ACMI		ACMI	AC	ACMI		ACM
Di	A	AI			ACMI		ACMI
Tri	ACMI				A		
Tetra							

A = Ames, C = Cytogenicity, M = Mammalian cell gene mutation, I = *in vivo*

Reproductive toxicity

Property	**Reproductive toxicity – NOAEL (mg/kg/day or mg/m^3) Rat unless otherwise indicated**						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	Oral 11 Dermal 625 Inha: 316mg/m^3	Ms, Oral <250 (5 week). Ms=760	♦	Oral: >125† ♦	**MS Oral: 720**		♦
Di	>612*	**Ms=Oral: 2200**			Oral: 1000‡		
Tri	♦						
Tetra							

* Testicular toxicity test only. This is the most sensitive reproductive toxicity endpoint for glycol ethers. All other endpoints would be expected to show a higher NOAEL.
† OECD 421; dose limited by maternal toxicity and no developmental or reproductive effects observed at this level. ‡ OECD 415 equivalent. Values in bold are from an OECD 416 2-generation study or equivalent.

♦ No adverse testicular effects seen in repeated-dose studies. Data not added for substances with full 2-generation studies available.

Property	**Developmental toxicity (NOAEL mg/kg/day or mg/m^3) Rat unless otherwise indicated**						
Number of EO units	Alcohol chain length						
	Methyl	Ethyl	Propyl		Butyl	Pentyl	Hexyl
			n-	iso			
Mono	Oral 11 Dermal 48 Inha: 316	Oral 23 Rt, Rb: Inha 190	Inha: 1730**, Rb>2200	Oral: 125†	Oral: 100** Inha: Rb 490, Rt >980**		Rt, Rb. Inha: >490§
Di	Oral 200, Rb dermal 250*	Oral >1000 Dermal 1400			Oral >633 Rb Dermal >1000		>Oral 1000 ††
Tri	Rt, Rb >1000**				Rb>1000‡		
Tetra							

* Rabbit – marginal effects at 50 mg/kg
** No developmental effects seen up to levels of marked maternal toxicity.
† OECD 421.
†† OECD 422.
§ Close to saturated vapour pressure. Maternal toxicity seen.
‡Chernoff Kavlock assay.

Endpoints for which read-across is used in REACH registration dossiers

Substance	Endpoint	Substance used to fill gap
DEGME	Mutagenicity –cytogenicity	DEGBE
	Mutagenicity – mammalian cell gene mutation	DEGBE
	Toxicity to reproduction (2-generation study)	DEGEE
TEGME	Toxicity to reproduction (2-generation study)	DEGEE
Methyl heavies	Melting point	TEGME
	Boiling point	TEGME, TetraEGME
	Density	TEGME, TetraEGME
	Vapour pressure	TEGME, TetraEGME
	Partition coefficient	TEGME, TetraEGME
	Water solubility	TEGME
	Surface tension	TEGME
	Flash point	TEGME
	Auto ignition point	TEGME
	Dissociation constant	TEGME, TetraEGME
	Viscosity	TEGME, TetraEGME
	Biodegradation	TEGME, TetraEGME
	Acute toxicity to fish	TEGME, TetraEGME
	Acute toxicity to invertebrates	TEGME, TetraEGME
	Acute toxicity to algae	TEGME
	Toxicity to microorganisms	TEGME, TetraEGME
	Acute oral toxicity	TEGME, TetraEGME
	Acute dermal toxicity	TEGME
	Skin irritation	TEGME, TetraEGME
	Eye irritation	TEGME, TetraEGME
	Skin sensitisation	Brake fluid mixture
	Repeated-dose toxicity – oral route	TEGME
	Repeated-dose toxicity – dermal route	TEGME
	Mutagenicity – bacterial gene mutation	TEGME
	Mutagenicity – cytotoxicity	Brake fluid mixture
	Mutagenicity – mammalian cell gene mutation	TEGME
	Toxicity to reproduction (2-generation study)	DEGEE
	Developmental toxicity	TEGME
DEGEE	Surface tension	DEGME, TEGEE, EGEE
	Acute algal toxicity	DEGBE

Substance	Endpoint	Substance used to fill gap
TEGEE	Partition coefficient	TEGME, TEGBE
	Surface tension	TEGME, TEGBE
	Flash point	TEGME, TEGBE
	Auto ignition point	TEGME, TEGBE
	Acute toxicity to algae	TEGME, TEGBE
	Acute dermal toxicity	TEGME, TEGBE
	Skin irritation	TEGME, DEGEE
	Eye irritation	TEGME, DEGEE
	Skin sensitisation	Brake fluid mixture
	Repeated-dose toxicity – oral route	TEGME
	Repeated-dose toxicity – dermal route	TEGME
	Mutagenicity – bacterial gene mutation	TEGME
	Mutagenicity – cytotoxicity	Brake fluid mixture
	Mutagenicity – mammalian cell gene mutation	TEGME
	Toxicity to reproduction (2-generation study)	DEGEE
	Developmental toxicity	TEGME
EGnPE	Toxicity to reproduction (2-generation study)	EGBE
EGiPE	Surface tension	EGnPE
	Acute oral toxicity	EGnPE
	Acute dermal toxicity	EGnPE
	Acute inhalation toxicity	EGnPE
	Eye irritation	EGnPE
	Repeated-dose toxicity – oral route	EGnPE
	Mutagenicity – mammalian cell gene mutation	EGBE
	Toxicity to reproduction (2-generation study)	EGBE
	Developmental toxicity	EGnPE
DEGBE	Surface tension	DEGME, EGBE
	Toxicity to reproduction (2-generation study)	EGBE
TEGBE	Eye irritation	Butyl heavies
	Skin sensitisation	DEGBE, brake fluids mixture
	Repeated-dose toxicity – oral route	TEGME, DEGBE
	Repeated-dose toxicity – dermal route	TEGME
	Mutagenicity - cytogenicity	TEGME, DEGBE
	Mutagenicity – mammalian cell gene mutation	DEGBE, brake fluids mixture
	Toxicity to reproduction (2-generation study)	EGBE, DEGEE
	Developmental toxicity	TEGME

Substance	Endpoint	Substance used to fill gap
Butyl heavies	Melting point	TEGBE
	Boiling point	TEGBE
	Density	TEGBE
	Vapour pressure	TEGBE
	Partition coefficient	NLP Bu heavies
	Water solubility	TEGBE
	Surface tension	TEGBE
	Flash point	TEGBE
	Auto ignition point	TEGBE
	Dissociation constant	TEGBE
	Viscosity	TEGBE
	Acute toxicity to fish	NLP Bu heavies
	Toxicity to microorganisms	TEGBE
	Acute oral toxicity	TEGBE, TetraEGBE, NLP Bu heavies
	Acute dermal toxicity	TEGBE
	Skin irritation	TEGBE, TetraEGBE, NLP Bu heavies
	Skin sensitisation	TEGME, DEGBE
	Repeated-dose toxicity – oral route	TEGME, DEGBE
	Repeated-dose toxicity – dermal route	TEGME
	Mutagenicity – bacterial gene mutation	TEGBE
	Mutagenicity – cytotoxicity	DEGME, Brake fluid mixture
	Mutagenicity – mammalian cell gene mutation	TEGME, DEGBE
	Toxicity to reproduction (2-generation study)	EGBE
	Developmental toxicity	TEGME, EGBE
NLP Bu heavies*	Melting point	TEGBE
	Boiling point	TEGBE
	Density	TEGBE
	Vapour pressure	TEGBE
	Water solubility	TEGBE
	Surface tension	TEGBE
	Flash point	TEGBE
	Auto ignition point	TEGBE
	Dissociation constant	TEGBE
	Viscosity	TEGBE
	Toxicity to microorganisms	TEGBE
	Acute dermal toxicity	TEGBE
	Skin sensitisation	TEGME, DEGBE
	Repeated-ose toxicity – oral route	TEGME, DEGBE
	Repeated-dose toxicity – dermal route	TEGME
	Mutagenicity – bacterial gene mutation	TEGBE
	Mutagenicity – cytotoxicity	DEGME, Brake fluid mixture
	Mutagenicity – mammalian cell gene mutation	TEGME, DEGBE
	Toxicity to reproduction (2-generation study)	EGBE
	Developmental toxicity	TEGME, EGBE

Note that no read-across is required to fulfil the requirements of the EGBE dossier.
* NLP Bu - No longer polymer butyl

References

The great majority of the information used in this category justification was sourced from the REACH registration dossiers for the substances (Note: This was prepared by Chemsage, Global Product Stewardship on behalf of the Glycol Ethers REACH consortia). The information is available from the dossier dissemination pages on the ECHA website. It was supplemented by information from the following sources:

Bingham E, Cohrssen B, Powell CH, eds. 2001. Patty's Toxicology, 8 Volume + Index Set, 5th Ed. Wiley and Sons, 9008 pp.

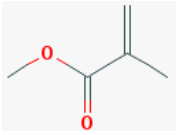
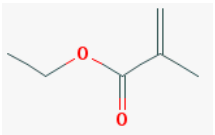
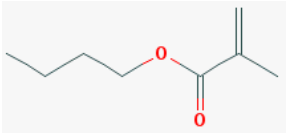
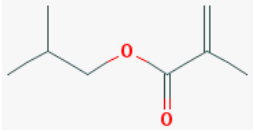
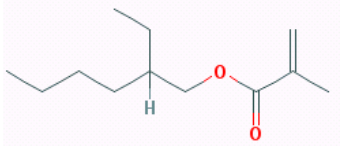
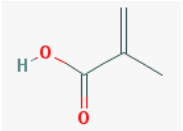
ECETOC. 2005. The Toxicology of Glycol Ethers and its Relevance to Man (Fourth Edition). Technical Report 95, vol I. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.

ECETOC. 2005. The Toxicology of Glycol Ethers and its Relevance to Man (Fourth Edition). Technical Report 95, vol II, Substance Profiles. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.

B.5. Lower alkyl methacrylate esters

The short-chain C1-C8 alkyl esters represent a commercially important group of chemicals that are used as monomers in the manufacture of plastic articles and a wide range of industrial, professional and consumer polymer based products. Methyl methacrylate (MMA) is the largest volume chemical and consequently benefits from a comprehensive data set of mammalian and environmental toxicity studies as well as extensive toxicokinetic studies (physiologically-based pharmaco kinetic [PBPK] investigations into the lead effect following inhalation and comprehensive metabolic fate studies in rodents). Since the other C2-C8 esters are of lower production volume and are typically controlled in the occupational setting by reference to MMA, the most volatile ester, they have incomplete data sets. In 2000 and in anticipation of the data requirements for REACH, extensive metabolism studies were commissioned on the C2-C8 esters to test the rationale for proposing a common metabolic fate and mode of action (MOA) thereby enabling a strategy of targeted testing and read-across between esters.

1. Category chemicals

Chemical name of category members	Abbreviation	EC-No.	CAS no.	Structure
Methyl methacrylate	MMA	201-297-1	80-62-6	
Ethyl methacrylate	EMA	202-597-5	97-63-2	
n-Butyl methacrylate	n-BMA	202-615-1	97-88-1	
Iso-Butyl methacrylate	i-BMA	202-613-0	97-86-9	
2-Ethylhexyl methacrylate	2-EHMA	211-708-6	688-84-6	
Reference chemical				
Methacrylic acid	MAA	201-204-4 79-41-4		

2. Hypothesis for the category approach

The members of the category are the lower alkyl esters of methacrylic acid with a carbon chain length of the alcohol side chain of 1 to 8 and, as a reference chemical, the common, primary metabolite, methacrylic acid. The table below shows the most important category members. Between the category members there are consistent trends regarding their physicochemical properties, toxicity and ecotoxicity and they share common metabolic pathways and a rapid metabolism. 2-EHMA, the C8 ester has been chosen as the upper limit of the category because it represents the largest ester with appreciable acute ecotoxicity and hence acts as a boundary for this endpoint. Larger esters with a longer alkyl side chain have water solubility which is so low that they are above the solubility cut-off for this endpoint.

3. Purity / impurities

Typical commercial grades of methacrylic acid and its basic alkyl esters are all high-purity chemicals of 98+ % purity with typical trace impurities of water and the corresponding alcohols. All substances are stabilised against spontaneous polymerisation by the addition of hindered phenols such as hydroquinone or hydroquinone monomethyl ether.

4. Scope of category approach

Consistent trends in physicochemical and toxicokinetic properties build the framework for the category approach. Albeit technically justified and possible it has not been used to substitute physicochemical test data since these data formed a robust basis for some of the correlations and it enabled outliers to easily be identified.

Where the category data have not been used to read across, because actual test data were available, read-across has been used to confirm consistency and trends in the data, thereby increasing the overall confidence in the database.

5. Endpoints

Where no specific reference for the data is given in this case study, the information was sourced from the REACH registration dossiers for the substances. The data are available from the dossier dissemination pages on the ECHA website.

Physicochemical data

Table B5-1: Carboxylesterase mediated hydrolysis of a methacrylate ester to MAA and the corresponding alcohol

Property	Value					
	MAA	**MMA**	**EMA**	**n-BMA**	**i-BMA**	**2-EHMA**
Physical state at 20°C and 101.3 kPa	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
Melting/freezing point [°C]	15.4-15.5	-48	<-75	-50	-35	<-50
Boiling point [°C]	162	100.36	118.2	163	155	227.6
Relative density [D 20°C/4°C]	1.01	0.94	0.91	0.90	0.88	0.88
Vapour pressure [hPa at 20 °C]	0.97	37	20	2.1	2.1	0.065
Water solubility [g/l at 20°C]	98	15.3	4.69	0.36 (25°C)	0.47	0.003
Partition coefficient n-octanol/water (log value)	0.93	1.38	1.87	3.0	2.95	4.95

As a rule, boiling point and octanol/water partition coefficient increase, and vapour pressure and water solubility decrease, with increasing molecular weight/volume (i.e. alkyl ester group) within the series. Methacrylate esters up to and including 2-EHMA may be considered to exert a significant vapour pressure and are sparingly soluble in water. Methacrylate esters with side chain groups larger than 2-EHMA are practically insoluble in water and exert little or no vapour pressure and are therefore excluded from this category.

Fate in the environment / degradation

Hydrolysis

Methacrylate esters are stable at neutral and acidic pH. Under alkaline conditions at pH 9 or 11, significant hydrolysis is observed with a trend to a decrease of the hydrolysis rate with increasing chain length and branching. This is partly due to increased steric hindrance and partly due to decreasing alkoxide stability during ester cleavage. Under normal environmental conditions, abiotic degradation by hydrolysis is not expected to play an important role in the degradation of methacrylates in the environment.

Photodegradation

Based on measured data for MMA and model calculations, photodegradation is expected to be rapid. Photodegradation in the atmosphere is expected to occur either by reaction with photo-chemically produced hydroxyl radicals or by reaction with ozone. The reaction half-lives for the atmospheric oxidation of the methacrylate esters by hydroxyl radicals range between 6.9 h for MAA, 7.0 h for MMA, 6.5 h for EMA, 5.7 h for n- and i-BMA and 4.4 h for 2-EHMA with a trend towards shorter half-lives with increasing molecular

weight. For the reaction with ozone an atmospheric half-life of approximately one day has been estimated for all esters.

Biodegradation

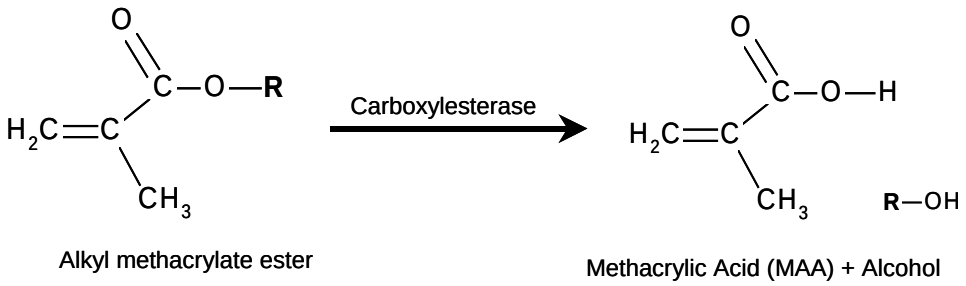
The test data with overall biodegradation rates of 74 to 94 % and more than 60 % degradation achieved within 10 days indicate that all of the substances within the category are rapidly biodegraded and completely mineralised.

Bioaccumulation

Model calculations based on logP for the smaller esters up to n- and i-BMA and the metabolite, MAA, indicate low bioaccumulation potential. For 2-EHMA, the largest member, data from a fish bioconcentration test indicates a low bioconcentration factor (BCF) of 37. All members of the category are non-bioaccumulative.

Metabolism and toxicokinetics

Figure B5-1: Carboxylesterase mediated hydrolysis of a methacrylate ester to MAA and the corresponding alcohol



Short-chain alkyl-methacrylate esters are rapidly hydrolysed by ubiquitous carboxylesterases (see table below, adapted from Jones, 2002). First pass (local) hydrolysis of the parent ester has been shown to be significant for all routes of exposure. For example, no parent ester can be measured systemically following skin exposure to EMA and larger esters, as the lower rate of absorption for these esters is within the metabolic capacity of the skin (Jones, 2002). Parent ester will also be effectively hydrolysed within the G.I. tract and within the tissues of the upper respiratory tract (particularly the olfactory tissue). Systemically absorbed parent ester will be effectively removed during the first pass through the liver (%LBF; see table below) resulting in their relatively rapid elimination from the body ($T_{50\%}$; see table below).

Table B5-2: Rate Constants for ester hydrolysis by rat-liver microsomes and predicted systemic fate kinetics following i.v. administration (adapted from Jones, 2002)

Ester	Rat liver microsomes (100 μg ml^{1}) V_{max} (nM min^{-1} mg^{-1})	K_m (μM)	CL (%LBF)	$T_{50\%}$ (min)	C_{max} (MAA) (mg L^{-1})	T_{max} (MAA) (min)
MAA	-	-	51.6%	-	-	-
MMA	445.8	164.3	98.8%	4.4	14.7	1.7
EMA	699.2	106.2	99.5%	4.5	12.0	1.8
i-BMA	832.9	127.4	99.5%	11.6	7.4	1.6
n-BMA	875.7	77.3	99.7%	7.8	7.9	1.8
HMA	376.4	34.4	99.7%	18.5	5.9	1.2
2EHMA	393.0	17.7	99.9%	23.8	5.0	1.2
OMA	224.8	11.0	99.9%	27.2	5.0	1.2

HMA – n-hexyl methacrylate; OMA – n-octyl methacrylate. Fate kinetics determined using the 'well-stirred' model; CL%LBF – Clearance as percentage removed from liver blood flow i.e. first pass clearance; $T_{50\%}$ - time taken for 50% of parent ester to have been eliminated from the body; C_{max} – maximum concentration of MAA in circulating blood; T_{max} – time in minutes to peak MAA concentration in blood.

Table B5-3: Summary of the results for the peak rates of absorption of MAA and alkyl methacrylate esters through rat and human epidermis

	Rat epidermis			Human epidermis		
Ester	**Peak rate of absorption (μg cm-2hr^{-1}) ±SEM**	**Period of peak absorption rate (hours)**	**% age of applied dose absorbed over x hours**	**Peak rate of absorption (μg cm-2hr^{-1}) ±SEM**	**Period of peak absorption rate (hours)**	**% age of applied dose absorbed over x hours**
MAA	23825±2839	0.5-4	93% / 24h	*812*	-	-
MMA	5888±223	2-8	46% / 16h	453±44.5	4-24	10% / 24h
EMA	*4421*	-	-	*253*	-	-
i-BMA	*1418*	-	-	*80*	-	-
n-BMA	1540±69	0-6	18% / 24h	76.7±9.8	0-24	2% / 24h
HMA	*147*	-	-	*25*	-	-
2EHMA	234±4.8	0-30	7.8% / 30h	22.7±3.7	3-24	0.6% / 24h
OMA	159±15	0-24	-	*7.8*	-	-

Note: The values in normal type were obtained experimentally, whilst those in italics, are predicted values based on statistical analysis (single exponential fit) of the experimental data.

Methacrylate esters can conjugate with glutathione (GSH) *in vitro*, although they show a low reactivity, since the addition of a nucleophile at the double bond is hindered by the alpha-methyl side-group (McCarthy and Witz, 1991; McCarthy et al, 1994; Tanii and Hashimoto, 1982). Hence, ester hydrolysis is considered to be the major metabolic pathway for lower alkyl-methacrylate esters, with GSH conjugation only playing a minor role in their metabolism, and then likely only when very high tissue concentrations are achieved.

Trends
Short-chain esters are absorbed by all routes. The rate of absorption decreases with increasing ester chain length. All esters are rapidly hydrolysed in local tissues as well as in blood by non-specific esterases. There is a trend towards increasing half-life of the ester in blood with increasing ester chain length (see table with half-life data in blood above). The primary metabolite, MAA, is cleared rapidly from blood in all cases.

Conclusions
C1 – C8 Methacrylate esters are readily absorbed by all routes and rapidly hydrolysed by carboxylesterases to methacrylic acid (MAA) and the respective alcohol. Clearance of the parent ester from the body is in the order of minutes. The primary metabolite, MAA, is subsequently cleared rapidly from blood and, as indicated by studies with MMA, this metabolism is by standard physiological pathways, with the majority of the administered dose being exhaled as CO_2. The other metabolites, short-chain alkyl alcohols, are all well-investigated HPV chemicals with similar metabolism.

Acute toxicity

Oral and dermal toxicity

All methacrylate esters are of low acute toxicity with the median lethal dose values (LD_{50}) being significantly greater than 2000 mg/kg by oral and dermal routes. Available data cover the whole range of the C1-8 category (oral: all category members, dermal: MMA, n-BMA) and potentially, with appropriate bridging studies, beyond the C8 range.

Reliability of the database: High.
Reliability of the prediction: High.

Inhalation toxicity

All members of the category are of low acute toxicity with the median lethal dose values (LD_{50}) being significantly greater than 5000 ppm by inhalation. Also due to decreasing vapour pressure with increasing molecular weight, acutely toxic concentrations for the butyl esters and above are associated with exposure to aerosol (above the density of saturated vapour).

Available data cover the whole range of the category and could be extended to other alkyl methacrylates within the C1-8 domain and potentially, with appropriate bridging studies, beyond the C8 range.

Reliability of the database: High.
Reliability of the prediction: High.

Irritation

Skin and eye irritation: All members of the category are weak to moderate skin irritants, particularly after exposure under occlusion for more than 4 hours. Eye irritation is either absent or very slight.

Respiratory irritation: The smaller members of the category up to n-BMA are respiratory irritants. All members of the category primarily affect the olfactory epithelium in the upper respiratory tract, a metabolically active tissue with high carboxylesterase activity. MMA and EMA have shown this effect after single exposures, in n-BMA it was found in a 28-day inhalation study in rats. Due to the low vapour pressure, lower absorption, and slower hydrolysis 2-EHMA, the largest member of the category, is not considered to be a respiratory irritant.

Available data cover the whole range of the C1-8 domain and potentially, with appropriate bridging studies, beyond the C8 range.

Reliability of the database: High.
Reliability of the prediction: High.

Sensitisation

Skin sensitisation

MMA and several other esters have been associated with skin sensitisation.

Table B5-4: Comparison of human incidence (Patch Test) data: Methacrylate esters

Subjects / Study type	MMA	EMA	n-BMA	References
Patients with dermatitis (suspected of (meth)acrylate allergy; no further details)	51/4221 1.2 %	16/2323 0.7 %	1/347 0.3 %	Schnuch, 1996
Patients with dermatitis (suspected of (meth)acrylate allergy; no further details)	9/1161 0.8 %	2/625 0.3 %	no data -	Schnuch, 1997
Patients with dermatitis with previous contact with (meth)acrylate (no further details)	17/352 4.8 %	11/246 4.4 %	2/331 0.6 %	Tucker and Beck, 1999
Patients with dermatitis (suspected of (meth)acrylate allergy; no further details)	20/271 7.4 %	18/243 7.4 %	6/243 2.5 %	Kanerva et al, 1997

MMA is the only category member for which LLNA (local lymph node assay) data are available. Other sensitisation test data exist in the form of a multitude of adjuvant-using (e.g. GPMT) and non-adjuvant tests (e.g. Buehler) for MMA and the higher esters. Cross-reactivity data indicate that cross-sensitisation may occur to a certain extent. In addition, human incidence data have been reported for MMA, EMA and n-BMA.

The data confirm that EMA and n-BMA are contact allergens in humans, although cross reaction with MMA cannot be excluded. Betts reviewed prevalence data for published clinical studies on contact allergy due to

MMA in humans (i.e. Peiler et al, 1996; Schnuch and Geier, 1994; Kiec-Swierczynska, 1996). In observing that there was a positive bias to inclusion of sensitised individuals in the test cohort used in these studies, thereby overstating actual prevalence, Betts concluded that MMA *"has only a relatively weak potential to cause the acquisition of skin sensitization"* (Betts et al, 2006).

Trend
Based on LLNA data in combination with human incidence/prevalence data, MMA is considered a skin sensitiser with a low potency. The incidence/prevalence data in patients suggest that EMA, the BMAs and 2-EHMA are less potent skin sensitisers than MMA.

Available data cover the whole range of the C1-8 domain and potentially, with appropriate bridging studies, beyond the C8 range.

Reliability of the database: High.
Reliability of the prediction: High.

Respiratory sensitisation

MMA is not considered a respiratory sensitiser and there is no concern from the literature regarding the other esters.

Repeated-dose toxicity

There are studies for most, but no all category members. For MMA oral (drinking water) and inhalation studies of up to 2-year duration have been performed. For n-BMA and 2-EHMA oral studies of up to 90-days are available and a 28-day inhalation study with n-BMA. For EMA and i-BMA no repeated-dose toxicity studies are available.

Local effects

Local irritation of the epithelia of the upper respiratory tract as a consequence of ester hydrolysis in metabolically active tissues like the olfactory epithelium of the nose has been observed in inhalation studies with the lower members of the category (MMA and nBMA). Although no repeat exposure studies exist for EMA, this effect has been observed in an acute study. This represents a common mode of action for local effects by inhalation for volatile alkyl methacrylates as well as other volatile organic esters.

Trend: This effect decreases with increasing molecular weight, lower water solubility and increasing octanol-water partitioning coefficient.

Reliability of the database: High.
Reliability of the prediction: High.

Table B5-5: Repeated-dose toxicity - NOAEC/NOAEL summary

	MAA	MMA	EMA	n-BMA	i-BMA	2-EHMA
Inhalation Local effects	100 ppm (358 mg/m^3)*	25 ppm 208 mg/m³ ** ***	---	310 ppm 1832 mg/m³	---	---
Inhalation Systemic effects	100 ppm 358 mg/m^3	500 ppm 1040 mg/m³	---	952 ppm 5626 mg/m³	---	---
Oral Systemic effects	---	164 mg/kg/d	---	120 mg/kg/d	---	120 mg/kg/d

* Direct irritant effect rather than ester cleavage.

** The departure point for the risk assessment is the SCOEL IOLV of 50 ppm, taking into account observations in humans.

*** No time-dependency or exacerbation of the local lesion was found in several studies between 6 hours and 2 years.

Systemic effects

The systemic effects observed for all members of the category as well as the metabolite, MAA, are mostly non-specific in nature, such as body and organ weight changes.

Trend: Considering the observed no-effect-levels and the molecular weights of the category members, there is a slight increase in toxicity on a molar basis, which would be consistent with non-specific toxicity.

Reliability of the database: High.
Reliability of the prediction: High.

Mutagenicity

The alkyl-methacrylate esters have been tested in a series of bacterial and mammalian tests *in vitro* and *in vivo* (see overview table below).

Table B.5-6: Mutagenicity overview– endpoint study availability

Ester	Ames	Gene mutation in mammalian cells	Chromosome aberrations *in vitro*	*In vivo* studies
MMA	√	√	√	√*
EMA	√	√	√	---
n-BMA	√	---	√	√
i-BMA	√	---	---	√
2-EHMA	√	√	√	---

* For MMA several *in vivo* studies are available covering gene and chromosomal mutations.

Except for the bacterial mutagenicity, studies are not available for all endpoints for all chemicals. However, all missing endpoints can be satisfied by read-across from other members of the category.

Clastogenicity has been observed occasionally with the smaller category members in some *in vitro* systems, including the induction of small colony mutants in the mouse lymphoma assay. However, that has not been confirmed in *in vivo* chromosome mutations assays. Except for the small colony mutant mentioned above, the category members have not been shown to induce gene mutations in mammalian cells.

Although the available dataset is not complete for all members of the category, it is sufficient for assessment purposes and could be extended to other alkyl methacrylates within the C1-8 domain and potentially, with appropriate bridging studies, beyond the C8 range. In summary, also taking into account the absence of concern from the alcohol metabolites, the basic alkyl methacrylates are considered to be non-mutagenic.

Reliability of the database: High.
Reliability of the prediction: High.

Carcinogenicity

Only MMA has been shown to be non-carcinogenic in chronic inhalation and drinking water studies in rodent species including the rat, mouse and guinea pig. For the other members the absence of mutagenic potential indicates no concern for carcinogenicity.

Although the available dataset is incomplete for all members of the category, it is sufficient for assessment purposes and could be extended to other alkyl methacrylates within C1-8 domain and potentially, with appropriate bridging studies, beyond the C8 range. In summary, the basic alkyl methacrylates are considered to be non-carcinogenic.

Reliability of the database: Moderate to high.
Reliability of the prediction: High.

Reproductive toxicity

Table B.5-7: Reproductive toxicity – available studies

	MAA	MMA	EMA	n-BMA	i-BMA	2-EHMA
Reproductive toxicity (Fertility screening data)				Rat, screening study		Rat, screening study
Reproductive toxicity (Fertility full study)		Rat, two-generation study				
Developmental toxicity (Screening data)				Rat, screening study		Rat, screening study
Developmental toxicity (Full study)	Rat, teratological study	Rat, rabbit, teratological study		Rat, rabbit, teratological study		

Fertility

Methyl methacrylate has been tested in a two-generation study in rats and n-BMA and EHMA tested in one-generation screening studies. No specific influence on fertility was observed and the non-specific effects observed parallels signs of general toxicity in the adults. As with the repeated-dose endpoint, a slight trend towards increasing toxicity on a molar basis is observed. This is supporting evidence for the non-specific nature of the toxicity.

Available data cover the whole range of the category and could be extended to other alkyl methacrylates within the C1-8 domain and potentially, with appropriate bridging studies, beyond the C8 range. In summary, there is no indication that the basic alkyl methacrylates have a specific impact on fertility.

Reliability of the database: Moderate to high.
Reliability of the prediction: High.

Developmental toxicity

As indicated in the table above, representative alkyl-methacrylate esters have been tested in rats and rabbits. For EHMA only screening data in rats are available. Specific malformations were not observed. Non-specific variations and changes in body weight were observed but only in the presence of significant signs of general toxicity in the dams.

Again, although the available dataset is incomplete for all members of the category, it is sufficient for assessment purposes and could be extended to other alkyl methacrylates within the C1-8 domain and potentially, with appropriate bridging studies, beyond the C8 range. In summary, the basic alkyl methacrylates are considered to be non-teratogenic.

Reliability of the database: Moderate to high.
Reliability of the prediction: High.

Ecotoxicity

Table B.5-8: Ecotoxicity – available studies

	MAA	MMA	EMA	n-BMA	i-BMA	2-EHMA
Acute fish toxicity	√*	√	√	√	√	√
Acute invertebrate toxicity	√*	√	√	√	√	√
Acute toxicity to aquatic plants	√*	√	√	√	√	√
Chronic fish toxicity	√	√				
Invertebrate daphnid reproduction toxicity	√	√	√	√	(QSAR)	√
Chronic toxicity to aquatic plants	√*	√	√	√	√	√

* Including marine species.

Representative alkyl-methacrylate esters have been tested in all standard trophic levels. Specific effects regarding trophic levels have not been observed – the data follow the baseline narcosis model. For MAA a number of marine species has been tested in addition. Throughout all trophic levels they were less sensitive than corresponding freshwater species. With high reliability of the database it was possible to identify deviating test data outside of those consistent trends which are now considered outliers and unreliable.

Available data cover the whole range of the category and could be extended to other alkyl methacrylates within the C1-8 domain and potentially, with appropriate bridging studies, beyond the C8 range.

Reliability of the database: High.
Reliability of the prediction: High (QSAR).

Conclusions
Lower C1-8 alkyl methacrylates represent a series of chemicals with clear structural and physicochemical trends. The metabolism and fate lend them to a common mode of action enabling data gaps to be filled by QSAR and read across between members (interpolation) on a molar basis. Similar trends across multiple endpoints provide confidence in the read-across predictions thereby avoiding the need for further testing. The trends observed for lower alkyl methacrylates with suitable bridging studies could be extended to other alkyl methacrylates beyond the C1-8 range, as long as potential concerns (alerts) for specific alcohol toxicity are addressed.

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B.6. Molybdenum compounds

The Molybdenum Consortium for REACH has prepared registration dossiers for 11 molybdenum substances (Molybdenum consortium, 2010). To avoid unnecessary (animal) testing, a comprehensive read-across concept has been developed by the consortium, which is based on the chemistry and composition of all substances and on three series of experimental studies:

- Water solubility of all compounds.
- Determination of the speciation of various molybdenum substances upon dissolution in aqueous solution by UV spectroscopic methods (Mitchell, 2009).
- *In vitro* bio-accessibility studies: assessment of the solubility of molybdenum substances in various artificial physiological fluids, i.e. phosphate buffered saline, artificial lysosomal fluid, Gamble's solution, artificial gastric fluid and artificial sweat (Ullmann and Odnevall Wallinder, 2009).

Based on the results of these studies the hypothesis for the analogue approach and its justification was formulated.

1. Hypothesis for the analogue approach

Upon dissolution in aqueous solutions at physiologically relevant concentrations and pH conditions, the only aqueous molybdenum species emerging from all considered molybdenum substances is the molybdate [MoO4]2- anion. This has been verified by the analysis of UV-spectra of solutions of various molybdenum substances. Thus, with respect to systemic toxicity, read-across between all molybdenum substances is generally justified. However, specifically for poorly soluble Mo species, read-across form highly soluble/highly bioavailable substances is likely to constitute a conservative overestimate.

2. Source chemicals

Based on the hypothesis above the source chemicals are highly soluble molybdate salts, e.g. sodium molybdate. Testing was performed with a high purity sodium molybdate if high quality studies were not available.

3. Purity/impurities

Possible influences of purities and impurities on the toxicity of molybdenum compounds were assessed for each compound separately based on the purity information of the respective compound.

4. Analogue approach justification

The water solubility of molybdenum substances varies from very soluble (e.g. sodium molybdate or ammonium dimolybdate) via moderately soluble (e.g. molybdenum trioxide) to almost insoluble (e.g. molybdenum metal, molybdenum dioxide). To substantiate this grouping concept, the solubility of molybdenum substances in various artificial physiological fluids was determined at a loading of 0.1 g substance per liter of fluid. Amongst other parameters, the fraction of dissolved material after 2h and 24h was determined. In all physiological media and at both time points, less than 10% of the material was dissolved for molybdenum (metallic) and molybdenum dioxide. In contrast, between 30-100% were dissolved of the other substances. Furthermore, for molybdenum compounds a large data base of published information on toxicokinetics both in humans and to a lesser extend animals is available that was carefully evaluated for this read-across approach. The results are summarised below.

Absorption

Based on numerous published human toxicokinetic studies, the toxicokinetics of molybdenum are well understood. Sophisticated biokinetic models for molybdenum uptake, distribution and elimination were developed. The modeled data indicate a highly efficient homeostatic mechanism over a wide range of intakes, suggesting diffusion rather than active transport as uptake mechanism (EBRC, 2010a).

Absorption via the oral route

Molybdenum absorption through the gastrointestinal tract occurs rapidly and almost completely (approx. 90% when given in water to fasted individuals), with little variation in absorption despite large variations in dose (i.e. between approx. 20-1400 µg/d orally). The influence of the food matrix on intestinal absorption (100% from water by comparison) has been investigated by co-administration with solid food (50% absorption) and black tea (~10% absorption), for example (EBRC, 2010a).

Adsorption after inhalation

Relevant animal or human data on inhalation absorption are not available for molybdenum substances. Due to the structure and nature of the respiratory tract, the inhalability and deposition of particles in various regions of the respiratory tract is dependent on particle characteristics such as size distribution and density, and will vary from species to species. Further clearance mechanisms may need to be considered. However, as a worst-case assumption, one may assume that soluble molybdenum substances are subject to complete systemic absorption after deposition in the respiratory tract.

Absorption via the dermal route

The dermal absorption of molybdenum is low to negligible, as has been shown in a guideline-conform *in vitro* percutaneous absorption study conducted under GLP using sodium molybdate (Molybdenum Consortium, 2010).

Distribution

Upon uptake, the highly soluble molybdate anions are widely distributed in the body. The highest Mo concentrations are found in kidneys, liver and bone. However, there is no apparent accumulation of Mo in animal or human tissues, and very little Mo seems to cross the placental barrier (Vyskocil and Viau, 1999).

Metabolism

Molybdenum is not subject to any metabolism in its true sense. Regardless of its original chemical speciation, it transforms rather quickly to molybdate anions upon dissolution. In this form, it is available via diet or drinking water, and represents the physiologically relevant Mo species. Once systemically available, molybdenum is stable in the anionic molybdate form and not subject to any changes in speciation or valence (EBRC, 2010a).

Excretion

The elimination of molybdenum (in the form of highly soluble molybdate anions) from plasma is rapid and predominantly via renal excretion (>80%) and only to a lesser extent via faeces (<10%) (EBRC, 2010a).

Conclusion

In conclusion, the relevant species present both under environmental and physiological conditions was determined to be sodium molybdate by making use of the characteristic UV-absorption spectra of different molybdenum species. Additional toxicokinetic information supports the fact that the molybdate ion is the only relevant species in this context for all considered molybdenum compounds. Data from the soluble compounds are therefore a relevant surrogate for molybdate toxicity. For the less soluble compounds dissolution kinetics in the relevant media may be considered as a factor modifying the release of molybdate ions.

5. Data matrix

The use of data for read across of molybdenum compounds is summarised in the table below (EBRC, 2010b). For individual substances the respective read-across data are characterised for the respective endpoints in more detail.

Substance / Formula	CAS:	Properties	Read-Across-Grouping Long-term effects	Read-Across-Grouping Acute effects
Roasted Molybdenite Concentrate (formula not available)	86089-09-0	soluble molybdenum substances water solubility above ca. 100 mg/l solubility in biological fluids 30-100% 'high bioaccessibility'	Grouped based on chemical similarity for long-term, local effects via inhalation (suspected carcinogenicity via inhalation). Grouped for all long-term systemic effects (all release MoO_4^{2-} ion).	Grouped for all acute effects (local and systemic).
Molybdenum Trioxide MoO_3	1313-27-5			
Sodium Molybdate Na_2MoO_4	10102-40-6			
Ammonium Dimolybdate $(NH_4)_2Mo_2O_7$	27546-07-2			
Ammonium Heptamolybdate $(NH_4)_6Mo_7O_{24}$	12027-67-7			
Ammonium Octamolybdate $(NH_4)_4Mo_8O_{26}$	12411-64-2			
Calcium Molybdate $CaMoO_4$	7789-82-4			
Iron Molybdate $Fe_2(MoO_4)_3$	13769-81-8			
Molybdenum (metal) Mo	7439-98-7	Poorly / hardly soluble molybdenum substances Water solubility well below ca. 10 mg/l Solubility in biological fluids well below 10% 'negligible bioaccessibility'	Grouped for all long-term systemic effects (all release MoO_4^{2-} ion) Conservative read-across to 'high bioavailability' group above.	Grouped for all acute effects (local and systemic) Conservative read-across to 'high bioavailability' group above.
Ferromolybdenum Slags (UVCB, formula not available)	84144-95-6			
Molybdenum Dioxide MoO_2	18868-43-4			

6. Conclusions per endpoint

Acute toxicity

Acute oral and dermal toxicity studies were available for soluble, moderately and poorly soluble molybdenum compounds and all values were very similar and above 2000 mg/kg bw. Similarily, acute inhalation toxicity studies did not reveal mortality with any of the compounds at the highest concentrations tested in inhalation toxicity studies.

Irritation/sensitisation

Soluble, moderately soluble and poorly soluble molybdenum compounds were not irritating to rabbit skin and eyes in standard tests and did not induce skin sensitisation in the guinea pig maximisation test.

Genotoxicity

In vitro genotoxicity tests for gene mutations in bacterial and mammalian cells as well as chromosomal aberrations in mammalian cells were performed with sodium molybdate and showed clearly negative results, thus provide strong evidence for an absence of concern for genotoxic effects of molybdenum substances. Since the substance tested represents a highly soluble molybdate, and all molybdenum substances regardless of their solubility, speciation and valence have been shown to transform rapidly to molybdate anions upon dissolution in aqueous media, these results can be read across to all other molybdenum substances without restriction. Because of their ubiquitous physiological presence in biota and/or their essential role in human physiology, the sodium/ammonium/calcium/iron moieties in some of the molybdenum substances are not considered to be of concern for genotoxicity.

Repeated-dose toxicity

With regard to repeated-dose toxicity testing, 13-week inhalation studies and 2-year carcinogenicity studies are available for molybdenum trioxide in rats and mice (NTP, 1997). No systemic toxicity was observed in the 13-week studies up to the highest concentration tested of 100 mg/m^3. Thus, this concentration of 100 mg/m^3 represents an unbounded NOAEC for both species. In addition, the 2-year inhalation carcinogenicity studies with MoO_3 in rats and mice were investigated for any non-neoplastic systemic effects, which were not observed. Therefore, the results of the 2-year inhalation studies were considered as supporting data for the derivation of a systemic NOAEC of 100 mg/m^3. As molybdenum trioxide is representative of a soluble molybdenum compound and represents in this respect a worst-case assumption, these data were used for the read-across to other molybdenum species. As no reliable oral repeated-dose toxicity studies were available for any of the molybdenum compounds, this study was also used in a first tier for route to route extrapolation for oral exposure. However the consortium was also aware that, a 90-day oral study with sodium molybdate in rats was already running and additional data would be available in the future.

Due to the low dermal absorption of sodium molybdate in an *in vitro* dermal absorption study of sodium molybdate, together with the evidence that at the pH of the skin molybdenum is available as molybdate anion, a dermal DNEL was not derived.

Repeated-dose local effects, carcinogenicity

In the NTP 2-year inhalation study with rats and mice mentioned above, an increased incidence of localised carcinoma/adenoma in the lungs was reported. The observed localised carcinoma/adenoma are considered secondary to local lung tissue inflammation.

A long-term inhalation DNEL for local effects, together with a respective classification as category 2 carcinogen was derived for molybdenum trioxide and roasted molybdenite concentrate (RMC) only, as there

is evidence that the observed increased rate of lung tumour formation in the NTP study with molybdenum trioxide is secondary to local lung tissue inflammation. This inflammation is considered to be a result of the acidic reaction of MoO_3 in the lung fluids. In aqueous media, MoO_3 molecules react with water and release protons according to the following equation: $MoO_3 + H_2O \rightarrow MoO_4^{2-} + 2\ H^+$.

A similar reaction is plausible for RMC, which consists of various forms of molybdenum oxides, with the main constituent being MoO_3. The water solubility (20°C) of MoO_3 is approximately 1 g/L, whereas RMC is soluble at 0.56 g/L at 20°C.

The other substances considered in the read-across approach are not capable of producing this acidic effect because of their chemical composition. Therefore, the derivation of a DNEL for long-term, inhalation local effects is only applicable to the substances roasted molybdenite concentrate and MoO_3.

Reproductive endpoints

In the NTP 90-day and 2-year inhalation and carcinogenicity study using molybdenum trioxide, no test substance-related changes in male and female reproductive organs were observed in either rats or mice up to the highest concentration tested. Additionally, no changes in sperm counts were observed in that study. Furthermore, the Consortium was aware that in the USA a 90-day oral rat study (OECD 408, 1998), with additional male and female reproductive toxicity parameters, using sodium molybdate as the test substance, was already underway and a developmental toxicity test was also already scheduled (OECD 408, 1998).

Therefore new data should shortly be available on these endpoints.

Environmental toxicity endpoints

The speciation of molybdenum in aqueous media as a function of pH and molybdenum concentration has been thoroughly investigated and reported in open literature. Under physiological conditions (pH > 6.5) the sole molybdenum(VI) species is the molybdate anion, $[MoO_4]^{2-}$ (Cruywagen, 1999; Cruywagen et al, 2002). Additionally, from an environmental point of view the significant Mo species is the simple $[MoO_4]^{2-}$ ion since this is the species which enters the cell in plants and animals (Stiefel, 2002; Pau and Lawson, 2002). Molybdenum compounds (e.g. molybdenum trioxide and polymolybdates) transform rapidly to the $[MoO_4]^{2-}$ ion under environmentally relevant test conditions (Greenwood and Earnshaw, 1984). For the Mo substances for which read-across is applied, UV-spectra of aqueous solutions indeed demonstrated that the only dissolved molybdenum species, originating directly from these substances, is molybdate.

These findings justify the implementation of a read-across strategy with results obtained in tests that were conducted with sodium molybdate for all relevant environmental endpoints, i.e. aquatic toxicity, sediment toxicity, terrestrial toxicity, toxicity to birds and toxicity to mammals. Sodium molybdate is a readily soluble substance, and yields molybdate ions upon dissolution.

Similarly the derivation of environmental fate data like adsorption/desorption coefficients and bioconcentration/bioaccumulation factors is based on measured Mo-levels, and reflect the properties of the molybdate anion.

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