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A Scheme for the Assessment and Definition of Tolerable Uncertainty in Read-Across for Toxicological Data Gap Filling

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Abstract

The transparency and explainability of uncertainties related to read-across predictions are critical for filling toxicological data gaps. As frameworks for evaluating read-across have become standardised, so has the identification and characterisation of the various types of uncertainty, particularly those related to chemical similarity. However, it has proven more challenging to assess overall uncertainty, particularly in defining what constitutes "tolerable" uncertainty. In this study, seven areas of uncertainty related to read-across were identified and their impact on read-across for two endpoints assessed; six related to aspects of chemical structure and properties, and a further one to uncertainty within the biological data used for read-across. The impact of uncertainty associated with these seven factors was related to ordinal categories. Examples of uncertainty assessment in read-across data gap filling, where different source analogues and the same target substances were evaluated, are provided for skin sensitisation and sub-chronic systemic toxicity. The resulting scheme, a generic tabular matrix, offers a flexible and adaptable approach for assessing uncertainties related to read-across predictions, particularly those from a single-source analogue and includes an overall uncertainty level for the read-across. Analysis of existing read-across predictions provides a means to define the level of tolerable uncertainty.

Keywords: read-across; characterisation of uncertainty; tolerable uncertainty; assessment scheme

Highlights

- Uncertainty in read-across assessments is categorised into seven criteria
- Read-across uncertainty has been characterised and the relative impact identified
- Assessment of (overall) uncertainty based on chemical structure and properties
- Simple and transparent template for uncertainty in read-across
- Tolerable uncertainty of the accepted read-across identified

1. Introduction

Computational approaches in toxicology cover a wide range of techniques to predict the adverse effects of chemicals. At the simplest level, structure-activity relationships (SARs) are applied through structural alerts; these methods become more complex with the integration and application of artificial intelligence, which relies on machine learning of large, chemically diverse datasets (Madden et al., 2020). This spectrum of methods is referred to as *in silico* new approach methodologies (NAMs) and, as such, are crucial for animal-free safety assessments (Westmoreland et al., 2022; Schmeisser et al., 2023). One of the most commonly used *in silico* NAMs, especially for regulatory submissions, is read-across (Rovida et al., 2020; ECHA, 2023).

As previously noted (Wohlleben et al., 2023), read-across to fill toxicological data gaps involves inferring similar biological effects—such as the presence or absence of harmful effects and possibly potency—from similar chemical substances. This fundamental principle makes it one of the most essential tools in computational, or *in silico*, toxicology (Kovarich et al., 2019). According to the European Chemical Agency (ECHA), read-across has been widely used in regulatory submissions related to chemical safety (ECHA, 2023). However, challenges remain in understanding its limitations and determining its acceptability as a replacement for animal tests (Ball et al., 2014). A key challenge is that the effectiveness of read-across depends on the proper definition and measurement of similarity, which vary depending on the toxicological context (Mansouri et al., 2024). Therefore, the main difficulty often lies in proving and justifying the similarity between substances to infer that the target molecule (the data-poor one) will exhibit similar, or predictably different, activity compared to the source molecule (the data-rich one). Compounding this issue is a known shortage of reliable and acceptable data-rich "source" molecules (Patlewicz et al., 2025). As a result, challenges have arisen in relaxing the boundaries of the similarity criteria needed to define chemical groupings, including both target and source analogues with appropriate experimental data. Specifically, Schultz and Cronin (2017) identified difficulties in identifying and evaluating the uncertainties associated with a particular read-across extrapolation as one of the key hindrances in the acceptance of predictions.

In most cases, chemical structure similarity is key to identifying one or more analogues (source molecules) for a target molecule that lacks data (Hagan et al., 2025). Exceptions to primarily relying on structural similarity can occur with complex mixtures (such as those with Unknown or Variable composition, Complex reaction products, or Biological materials (UVCBs)) (Zhou et al., 2025) or when biological similarity is considered (Vrijenhoek et al., 2022). Chemical structure similarity can be broken down into several measurable aspects, including metrics for similarity, shared functional groups, molecular scaffolds when applicable, and various physico-chemical properties (Schultz et al., 2015). While all aspects of structural similarity are valuable, those related to relevant toxicokinetic and

toxicodynamic considerations of the specific toxicological endpoint being assessed are most crucial. For example, in the case of read-across for skin sensitisation, similarity in protein reactivity is expected, whereas for repeated-dose toxicity, similarity in metabolic clearance might be the critical factor (Wohlleben et al., 2023).

There is copious guidance on how to perform read-across for toxicological data gap filling (see, for example: ECHA, 2008; OECD, 2025; EFSA Scientific Committee, 2025). However, despite several decades of assessing read-across predictions, there are few clear guidelines on how to determine if two molecules, or substances, are “sufficiently” similar, in a quantitative manner, to be acceptable for a particular purpose. ECHA’s Read-Across Assessment Framework (RAAF) provides some insight through its Assessment Elements (AEs) (ECHA, 2017). Still, it offers no specific definition of how structural similarity may be assessed. Whilst definitive descriptions of acceptable similarity are challenging to provide, there is an opportunity to characterise uncertainties in the read-across as a means to help identify acceptable similarity (Schultz and Cronin, 2017).

Most guidance on conducting toxicological read-across recommends or requires considering uncertainties (ECHA, 2008; OECD, 2025; EFSA Scientific Committee, 2025). In this context, and for this paper's purposes, the European Food Safety Authority (EFSA) definition of uncertainty as “*a general term referring to all types of limitations in available knowledge that affect the range and probability of possible answers to an assessment question*” is relevant (EFSA Scientific Committee, 2018a). It is accepted that uncertainties in risk assessment can be identified, characterised, and, where possible, quantified (EFSA Scientific Committee, 2025). This process can also be applied to read-across, where various frameworks identifying uncertainties have been published (see, for example: Wu et al., 2010; Blackburn and Stuard, 2014; Schultz et al., 2015) and later unified by Schultz et al. (2019). Specifically, regarding structural similarity in read-across, the elements of uncertainty that support it may be identified, characterised, and potentially quantified.

To apply the concept of uncertainty in supporting and evaluating read-across, most current guidance refers to achieving "tolerable” (or “acceptable”) levels of uncertainty (EFSA Scientific Committee, 2025). However, there is often confusion because there are limited or no clear ways to define such a level. The situation becomes more complex when considering that tolerable uncertainty should be defined within the problem formulation of a read-across, and levels of tolerable uncertainty will vary depending on the context. It is also accepted that if uncertainty is too high for a specific purpose, additional information and evidence must be provided (Schultz and Cronin, 2017; Pestana et al., 2021, 2025; Patlewicz et al., 2025) or the read-across may ultimately be deemed unfit for purpose and not accepted. Although tolerable uncertainty may not be explicitly defined for a read-across to be accepted, the uncertainty must be tolerable to the decision maker. It is the responsibility of the

decision maker to act upon that information. In this context, EFSA (2018a) describe this as “practical certainty”. EFSA (2018a) state that practical uncertainty should “sufficient for the practical purpose at hand” – with regard to this investigation this would be the acceptability of a read-across for a particular purpose. Recent evaluations of ECHA’s accepted read-across assessments (Patlewicz et al., 2024; Roe et al., 2025a, b; Schmitt et al., 2025) have clearly illustrated the types of read-across that have been accepted — those with tolerable uncertainty.

The goals of this investigation were fourfold. Firstly, to identify, characterise, and qualitatively determine the uncertainties related to the structural basis of read-across, including relevant aspects of chemical similarity metrics, essential functional groups, and molecular scaffolds, as well as related physico-chemical and other data, including the toxicological data read across. Secondly, to propose a scheme that includes a generic tabular matrix offering a flexible and adaptable approach for assessing uncertainties associated with read-across predictions, especially those from a single-source analogue. Thirdly, to demonstrate the usefulness of the method by applying the matrix and analysing two series of read-across examples to measure the various uncertainties related to a particular read-across prediction. Fourthly, to use the scheme to assess uncertainties to identify tolerable uncertainties in published read-across examples.

2. Methods

2.1 Identification of uncertainties in the definition of molecular similarity

Molecular similarity assessments can use either endpoint-independent or endpoint-specific chemical and/or biological information; these approaches align with unsupervised and supervised methods, respectively (Mansouri et al., 2024). Unsupervised chemical grouping relies on general similarity measures to find patterns and relationships without prior knowledge of the toxic endpoint of interest, and such techniques help generate hypotheses about toxicity. However, they may not be ideal for grouping compounds to allow read-across of an OECD test guideline study, i.e., based on toxicodynamic considerations. In contrast, supervised methods require endpoint-specific similarity measures, such as those relating chemical features to a particular biological activity. These methods are suitable for developing endpoint-specific hypotheses and building predictive models to assess new chemicals, such as the profilers in the OECD QSAR Toolbox (Schultz et al., 2022), and form the basis of the investigation in this study.

It is acknowledged that many uncertainties may be identified regarding toxicological read-across. Such uncertainties include those due to similarity measurements, experimental studies, and within- and

between-species effects, and non-standard uncertainties, as well as those related to the applicability of the experimental data to be read across (Schultz et al., 2019; EFSA Scientific Committee, 2025; OECD, 2025). This study focuses primarily on uncertainty related to chemical structure and molecular properties, as these are fundamental to the initial identification of read-across analogues. Additionally, well-defined and justified chemical similarity should encompass the molecular aspects of toxicodynamics (e.g., interaction at the molecular site of action) and toxicokinetics (e.g., systemic bioavailability and metabolite production). Besides chemical similarities, the availability and quality of toxicological data are also crucial for the acceptance of read-across. Understanding the uncertainties related to toxicological data is essential.

The application of read-across is often facilitated by a workflow. Patlewicz et al. (2018) proposed a unified generic workflow incorporating several familiar steps, namely decision context and data gap analysis, definition of an overarching similarity rationale, analogue identification and evaluation, data gap filling, ending with uncertainty assessment. The generic read-across workflow has served as the basis for regulatory guidance (EFSA Scientific Committee, 2025; OECD, 2025). The experience of the authors of the current investigation is that the most crucial uncertainties in the workflow are those from the identification and evaluation of analogues and data gap filling. The identification and evaluation of analogues is a process that involves comparison of the target and source molecule in terms of 2D structural parameters, which may dictate toxicodynamic effects, relevant phyisco-chemical properties, factors related to toxicokinetics and pertinent *in vivo* or NAM data. Filling the data gap relates to utilising appropriate data for a suitable analogue and its justification. Thus, for the purposes of identifying the most critical uncertainties related to read-across, those related to specific aspects of chemical similarity and data quality were evaluated. Based on the authors' knowledge of the read-across process and the identification of acceptable analogues, as noted, a total of seven relevant uncertainty factors related to the following were determined:

2.1.1 Metrics of chemical similarity

The metrics of similarity can be calculated using various approaches and methodologies. Usually, they consist of two components: first, a description of the molecules, which could be based on physico-chemical properties or structural descriptors, but more commonly on one of the sets of "fingerprints" that indicate the presence or absence of structural features in the molecule (Cereto-Massagué et al., 2015; Mellor et al., 2019). The second component is the algorithm used to calculate the similarity. The most commonly used method is the Tanimoto index, along with the Dice, Cosine, and Manhattan indices. Alternative approaches (for continuous descriptors) include using k-nearest neighbours, Euclidean distances, and others (Bajusz et al., 2015; Maggiora et al., 2014; Willet et al., 1998). Another

commonly reported metric is the molecular formula, which is expressed as a count of the elements in a compound.

It is acknowledged that similarity metrics are not comparable across different descriptor sets/fingerprints or calculation methods. Additionally, they are influenced by the methodology used and may not accurately reflect similarity, mainly when activity cliffs are not accounted for (Lester et al., 2023; Mellor et al., 2019). They are often used as a preliminary step when searching databases and require additional information to make a well-informed decision and provide a justification for a read-across analogue.

2.1.2 Definition of chemical class

Chemical classes may be defined, this is typically a manual process that can include classes based on functional groups, molecular scaffolds, whether molecules are linear or branched, the number and type of rings, and other factors (Muldoon et al., 2025). Chemical classes can also be categorised into established groups, such as those defined by the United States Environmental Protection Agency (US EPA) (US EPA, 2024) or through the OECD QSAR Toolbox (Dimitrov et al., 2016).

2.1.3 Molecular similarity relating to toxicodynamics

The role of toxicodynamics can be evaluated by comparing molecules based on their functional groups or, should the information be available, molecular (sub-)structure(s) that define the molecular initiating event (MIE). Similarities in toxicodynamics are often grounded in the appropriateness of the premise or hypothesis, which may include mechanistic probability or plausibility, understanding the chemical mechanism of action, biological mode of action, or adverse outcome pathway. For local adverse effects, these include functional groups or extended molecular fragments that align with the molecular initiating event of appropriate Adverse Outcome Pathways (AOPs) (Cronin et al., 2017). These are often reactive functionalities, such as those involved in protein binding (Enoch et al., 2011), which are related to skin sensitisation and clastogenesis, or in DNA binding (Enoch and Cronin, 2010), which are associated with mutagenicity. When the MIE is known, typically, to ensure a conservative read-across, the source molecule should have similar or greater activity. Thus, for an endpoint associated with binding to DNA or proteins, the read-across analogue should be as reactive as, or more reactive than, the target.

For longer-term, multiple-dose effects (i.e., 90-day oral repeated-dose toxicity or developmental toxicity), coverage of AOPs is less comprehensive. Toxicodynamic uncertainties often require maximising the number of identical structural components between the target compound and the source chemical, supported by appropriate test data. Similarity in toxicodynamics may also be supported by receptor-binding similarity (Wu et al., 2023a), as well as *in vitro* and other NAM data,

208 including those from the -omics technologies (Barnett et al., 2025; de Abrew et al., 2022; Escher et al.,
209 2019; 2022; Pestana et al., 2021; Ross et al., 2025).

210 #### 2.1.4 Molecular similarity relating to toxicokinetics: bioavailability

211 For subchronic toxicity or repeat dose effects, which may be non-lethal, similarity in systemic
212 bioavailability of the molecule is often required for consideration. As bioavailability is linked to
213 clearance, functional groups that control this process should be assessed (e.g., Boyer et al., 2007; Wu
214 et al., 2023b). Generally speaking, to ensure a conservative read-across, the source molecule should
215 be at least as bioavailable as, or more bioavailable than, the target. Similarity in toxicokinetics may
216 also be supported by *in vitro* and other data (Laroche et al., 2018).

217 #### 2.1.5 Molecular similarity relating to toxicokinetics: the formation of common metabolites
218 or degradants

219 The formation of a common metabolite, or degradation product, is a common justification for read-
220 across arguments (Ball et al., 2014; ECHA, 2017; Patlewicz et al., 2025; Schultz et al., 2015). In addition,
221 common reactive metabolites may also be necessary (see Kalgutkar et al., 2005). It should be noted
222 that the rate of formation of the metabolite/degradant in the source molecule should be equivalent
223 to, or faster than, the target molecule. In addition, such a read-across hypothesis can be applied to
224 dissimilar molecules, so the other elements of similarity may be expected to be more uncertain.
225 Similarity in metabolite or degradant formation and the rate of formation may also be supported by
226 *in vitro* and other data (Yordanova et al., 2021).

227 #### 2.1.6 Physico-chemical properties relating to toxicokinetics

228 Similarity can be assessed based on relevant physico-chemical and molecular properties, such as
229 molecular weight, the logarithm of the octanol-water partition coefficient (log P), aqueous solubility,
230 vapour pressure, Henry's law constant, and melting and boiling points. Other ADME properties, such
231 as uptake from the gut and skin absorption, may also be considered. While experimental data and
232 values should be preferred over calculated ones, the difference between the target and source values
233 is meaningful (Pestana et al., 2025). Data should be taken from the same methodology or estimation
234 method to avoid further propagation of errors. Thus, in this scheme, uncertainty is minimally affected
235 by whether physicochemical properties are measured or calculated. The properties selected should
236 be relevant to the toxicological endpoint, such as dermal absorption for skin sensitisation, oral
237 absorption for repeated dose toxicity via gavage, and volatility for respiratory effects, etc.

238 #### 2.1.7 Toxicological data quality

The quality and reliability of read-across toxicology data are crucial in determining their acceptance (Schultz and Cronin, 2017). At a minimum, data for the source chemical should meet the quality and reliability requirements necessary to fill the data gap. For example, to fill a data gap for regulatory assessment, such as hazard identification, the data should typically be generated in accordance with OECD Test Guidelines and under Good Laboratory Practice (GLP) conditions.

It should be recognised that data quality assessment is itself subjective and prone to uncertainty and bias (Przybylak et al., 2012). Several schemes exist to evaluate the quality of toxicity data, with the most widely used described by Klimisch et al. (1997) and formalised, in part, within the ToxRTool (Schneider et al., 2009). Other viable evaluation schemes include Criteria for Reporting and Evaluating Ecotoxicity Data (CRED) (Moermond et al., 2016) and the Science in Risk Assessment and Policy (SciRAP) approach (Molander et al., 2015). To reduce variability in the assessment of data quality, consistent criteria should be applied which are relevant to the context of the read-across and endpoint, as defined by Przybylak et al. (2012)

2.2 Defining uncertainty in read-across on an ordinal scale

The aspects of uncertainty outlined in Section 2.1 were defined and described in terms of their importance. Uncertainty was measured with respect to:

i) For each aspect of uncertainty, relevant quantifiable criteria were defined.

ii) The levels of uncertainty were established using the criteria described by EFSA relating to the definition, description and quantification of uncertainty (EFSA Scientific Committee, 2018b). These were placed on an ordinal scale (very low, low, moderate and high).

iii) Relevant classifications of uncertainty were identified for the read-across process.

iv) For each aspect of uncertainty related to read-across, criteria are proposed concerning the information, data, or chemical property considered, i.e. those that are associated with a particular level of uncertainty.

v) The impact of uncertainties on the assessment conclusion (as termed by EFSA Scientific Committee, 2018b), i.e., the read-across assessment to fill a data gap, was evaluated. The goal here is to identify key uncertainties. The impact varied for the toxicological endpoints considered.

With regard to evaluating the impact of uncertainties, areas of uncertainty with high impact are those that were considered to be critical in determining the similarity between target and source molecules

for endpoint-specific read-across. Low impact uncertainties are those that, although they need to be considered in the overall assessment, are not regarded as primary drivers of toxicity. High impact uncertainties are essential in determining the overall level of uncertainty. A final impact level of “no impact" indicates that the uncertainty is not relevant for the read-across. The impacts were assessed according to the authors’ knowledge and state-of-the-art of read-across. Impacts are implicitly context and endpoint dependent.

2.3 Analysis of accepted read-across and definition of overall uncertainty

Read-across for data gap filling was assessed for the following two toxicological endpoints:

i) Skin sensitisation, for example, as indicated by the results of the local lymph node assay. Here, an essential aspect of chemical similarity relates to the molecule's ability to bind to immunoprotein covalently (or not) and to have a similar dermal absorption profile (Wareing et al., 2017).

ii) Sub-chronic systemic toxicity, as represented by an outcome, such as a no observed adverse effect level (NOAEL), of a repeated dose rodent assay (Schultz and Cronin, 2017).

The similarities between the target and source molecules were assessed, and uncertainty was determined based on the results from the factors identified in Sections 2.1 and 2.2. This was done for read-across scenarios deemed “acceptable.” Additionally, other read-across cases where molecular similarity was not sufficient to support read-across were noted.

The overall level of uncertainty for the read-across was assessed qualitatively (i.e., ordinal classification), relating in part to the recommendations in EFSA’s guidance (EFSA Scientific Committee, 2025) and others (Pestana et al., 2021, 2025). This was done by interpreting the uncertainty levels in relation to their impact on the endpoint. Except in rare cases, overall uncertainty was not considered greater than the highest individual uncertainty. Overall uncertainty could be lower than the highest individual uncertainty if that uncertainty's impact was low or minimal.

2.4 Determining uncertainty in published read-across predictions to identify tolerable uncertainty

One of the largest published collections of read-across predictions is that used in the safety assessments of fragrance materials compiled by the Research Institute of Fragrance Materials (RIFM) (Api et al., 2015). Although RIFM’s read-across assessments are not intended for regulatory use, they are carefully curated by staff, including toxic endpoint specialists, who complete a standard read-

across justification template. During the internal review process, each proposed read-across undergoes a tiered review by multiple groups of chemistry and toxicology experts.

To evaluate the usefulness of the proposed scheme to determine uncertainty, the “read-across justification” section of molecular pairings (a target and a source substance) and relevant endpoint sections where key data are reported in the Fragrance Materials Safety Assessments were analysed. All assessments were published within the last five years and are accessible through the Fragrance Material Safety Assessment Center (https://fragrancematerialsafetyresource.elsevier.com/).

After evaluating both individual and overall uncertainties for the published fragrance material read-across assessments, the uncertainties were deemed "tolerable." This established the benchmark for defining tolerable uncertainty, meaning the maximum level of uncertainty was the highest acceptable level for approving the read-across assessments.

3 Results

In this investigation, the authors identified the main areas of uncertainty in read-across, focusing on the structural basis of these factors and the toxicological data used to fill data gaps. The work is based on the premise that as the proportion of identical structural features between target and source molecules decreases, the need to evaluate various forms of chemical similarity increases to understand these differences. We propose a scheme to categorise these uncertainties qualitatively, which references the terminology used by EFSA (EFSA Scientific Committee, 2018b) and the ECHA RAAF assessment outcomes (AOs) (ECHA, 2017). Subsequently, read-across scenarios are evaluated for uncertainty, and a method for providing a qualitative overall uncertainty value is proposed. The intent is that all information and uncertainties outlined in Section 3 be addressed flexibly and adapted to the specific endpoint being read across and the context in which it is applied. Our goal is to develop such a scheme, provided that the adaptations are documented, to be appropriate for the toxicological context being scrutinised.

3.1 Chemical identification

As is consistent with numerous read-across frameworks, evaluating uncertainties in a proposed read-across requires accurate identification of both the target and source substances (Patlewicz et al., 2018). We have observed that structures that, where necessary, include details of isomerism are the most reliable form of chemical identification for analysing the structural factors that influence uncertainty. It is crucial to ensure that the names, which often have multiple options, and the CAS

registry number, which is usually one or none, match the structure. In our scheme, SMILES notation, which is frequently critical for *in silico* modelling, is the least essential chemical identifier, as multiple SMILES can represent the same substance..

3.2 Identification of uncertainties in the definition of structural similarity

A total of seven areas of uncertainty related to read-across were identified and are described in full in Section 2.1. Six of these concerned aspects of chemical structure and properties. The seventh involved uncertainty within the biological data that are to be read across. The authors contend that these seven criteria are sufficient to cover the main aspects of uncertainty in the read-across approaches as currently described by regulatory agencies such as EFSA (EFSA Scientific Committee) as well as the OECD (OECD, 2025).

3.3 Defining uncertainty in read-across

3.3.1 Determination of uncertainty

The uncertainty associated with the seven criteria described in Section 2.1 was assessed. In this case, an ordinal classification with four levels of uncertainty is used. These classifications (very low, low, moderate, and high) are explained and mapped onto EFSA's "Approximate probability scale" (Table 2; EFSA Scientific Committee (2018b) and the AOs from the ECHA RAAF (Table 2, ECHA (2017)), as shown in Table 1.

Table 1. The four ordinal terms proposed in this study to evaluate uncertainty in read-across are mapped onto the terms suggested by EFSA and utilised within the ECHA RAAF.

Uncertainty term proposed in this study	Relevant subjective probability range taken from Table 2, EFSA Scientific Committee (2018b)	Equivalent ECHA RAAF AO (Table 2, ECHA (2017))
Very low	• Greater than 95%	Score = 5
Low	• 90 - 95%	Score = 4-5
Moderate	• 66 - 90%	Score = 2-3
High	• Less than 66%	Score = 1-2

3.3.2 Defining the levels of uncertainty

The seven uncertainty criteria described in Section 2.1 were defined in terms of varying uncertainty levels, ranging from very low to high, using the definitions of uncertainty outlined in Table 1. These

criteria for measuring uncertainty are listed in Supplementary Information Table S1. The criteria are proposed based on the authors' knowledge, with an attempt to align with the state of the art and understanding in similarity and data quality assessment to support read-across, for instance as proposed by the EFSA Scientific Committee (2025). It is intended that the criteria should be flexible and adapted to allow incorporation of new knowledge as it becomes available. Each criterion is designed to enable meaningful assessment of uncertainty using simple aspects of chemical structure, such as similarity measures, chemical class, the presence of functional groups that affect toxicity or metabolic clearance, property similarities, and the quality of the data. Of the four uncertainty levels, very low and low are the most significant – very low indicates very high similarity, such as a salt or a one-carbon difference between the target and sources. Low uncertainty indicates reasonable similarity, while moderate uncertainty reflects a more relaxed consideration of uncertainty. Defining high uncertainty (which combines various EFSA probability terms, as listed in Table 1) is unlikely to be acceptable in any situation.

For metrics of chemical similarity, definitive values to categorise uncertainty for similarity levels are not provided. This is because different calculation methods and descriptors or fingerprints can yield different results (Mellor et al., 2019), so it is up to the assessor's interpretation.

3.3.3 Impact of uncertainty on the overall decision

Understanding the impact of uncertainty on decisions is a crucial step in assessing its influence. This impact must also be communicated clearly and unambiguously (EFSA Scientific Committee, 2018a, b). In the context of read-across, the "decision" refers to the confidence in the ability of the read-across to address a data gap. While this differs from EFSA's process of making an overall risk assessment decision, the same principle(s) can be applied. Additionally, considering the effect of individual uncertainties can help organise the overall uncertainty assessment in a read-across, indicating that uncertainties with a high impact should be prioritised. In contrast, those with a lower impact may be less significant to the overall evaluation.

The impact of each uncertain area on the overall assessment conclusion, such as the read-across assessment used to fill data gaps, was evaluated. Each of the seven uncertainty factors has a different level of impact, categorised as none, low, moderate, or high, as shown in Tables 2a and 2b for the two toxicological endpoints considered (skin sensitisation and repeated dose toxicity respectively).

The "sensitivity" of each of the uncertainty factors was considered for read-across as a whole and noted in Tables 2a and 2b. All factors were considered to have potentially high impact with the exception of metrics for chemical similarity. The definition of impact in areas of uncertainty for skin

sensitisation and repeated dose toxicity involves different aspects of impact. It is expected that each toxicological endpoint will have a unique set of impacts. Thus, the sensitivity towards read-across is associated with the relative "magnitude" of the factor, which is specific to a particular endpoint and could be adapted to the context, e.g., to account for metabolically activated skin sensitisers, or chronic toxicity with a specific mode of action. To achieve the overall impact on the uncertainty conclusion, the endpoint-specific magnitude may be used to counter the sensitivity, i.e., whilst an uncertainty factor may have the potential for high impact in the overall read-across process, impact may be reduced for a particular endpoint, as demonstrated in Tables 2a and 2b.

For skin sensitisation (Table 2a), the most significant impact on the magnitude of uncertainty related to read-across concerns molecular similarity in toxicodynamics, specifically whether the source molecule exhibits the same mechanism of reactivity and the same or greater rate of reactivity than the target molecule. Reactivity is currently well understood through the presence of functional groups (Enoch et al., 2011) and is considered to be the fundamental driving force for skin sensitisation (Wareing et al., 2017). It can also be represented by *in chemico* data (Alépée et al., 2023) or reactivity estimates from quantum chemical calculations (Enoch and Roberts, 2013). Uncertainty arises when a biotic or abiotic step is required to form the reactive species (Yordanova et al., 2021; 2024); this has a significant impact, especially when transformation products involve directly reactive molecules, but is not relevant and can be disregarded for others. Uncertainty regarding chemical properties related to, or directly assessing, skin penetration is less critical than overall reactivity as skin penetration, *per se*, is required but not a crucial driver of skin sensitisation. Therefore such properties are considered to have a moderate impact. Variations in skin penetration are acceptable, provided that the source molecule exhibits equal or greater skin penetration than the target (Gilmour et al., 2020). The impact of uncertainty in the chemical class is low, as in skin sensitisation, read-across depends more on reactivity, which can be independent of chemical class. As noted above, the impact of chemical similarity metrics is low due to inconsistent overall scores.

The magnitude of impact of individual uncertainties in repeat dose toxicity is significant for aspects of toxicokinetics, especially those related to molecular clearance (Table 2b). This also indicates that uncertainty related to toxicokinetic properties and chemical class is similarly high (Date et al., 2020). Uncertainty in toxicodynamics will be minimal unless a specific mechanism of action, such as a pesticidal mechanism, is identified and characterised.

The purpose of considering the impact of individual uncertainties on the read-across assessment conclusion is to ensure flexibility in assessing each of the uncertainty sources and to support adaptability across the various endpoints typically considered in a robust safety assessment (see Tables 2a and 2b). While the effects can be generalised, it is acceptable to modify the influence with

appropriate, context-specific justification. Thus the user or evaluator of a read-across assessment is encouraged to update and adapt the impact in accordance with existing knowledge and the state-of-the-art.

434 Table 2a. Overall impact of the seven types of uncertainty identified in Section 3.2 on data gap filling through read-across for skin sensitisation.

Source of Uncertainties in Read-Across to Impacting on the Overall Uncertainty (Outcome Statement)	Sensitivity of Uncertainty to Read-Across	Magnitude of Uncertainty Relating to Skin Sensitisation	Overall Impact on the Uncertainty of the Final Outcome (Read-Across for Data Gap Filling for Skin Sensitisation)
Qualitative impact of metrics of chemical similarity on uncertainty in read-across for data gap filling (for skin sensitisation)	Low	Low	Low impact
Qualitative impact of chemical class on uncertainty in read-across for data gap filling (for skin sensitisation)	High	Low	Low impact
Qualitative impact of molecular similarity relating to toxicodynamics on uncertainty in read-across for data gap filling (for skin sensitisation)	High	High	High impact
Qualitative impact of molecular similarity relating to toxicokinetics on uncertainty in read-across for data gap filling (for skin sensitisation)	High	Where relevant, low impact	Low impact
Qualitative impact of molecular similarity relating to the formation of common metabolites or degradants on uncertainty in read-across for data gap filling (for skin sensitisation)	High	Where relevant, high impact; where not applicable, no impact (and need not be assessed)	When metabolism is relevant to skin sensitisation – high impact, otherwise – low impact
Qualitative impact of chemical properties relating to toxicokinetics on uncertainty in read-across for data gap filling (for skin sensitisation)	High	Moderate	Moderate impact
Qualitative impact of toxicological data quality chemical properties on uncertainty in read-across for data gap filling (for skin sensitisation)	High	High	High impact

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437 Table 2b. Overall impact of the seven types of uncertainty identified in Section 3.2 on data gap filling through read-across for repeat dose toxicity.

Source of Uncertainties in Read-Across to Impacting on the Overall Uncertainty	Sensitivity of Uncertainty to Read-Across	Magnitude of Uncertainty Relating to Repeat Dose Toxicity	Overall Impact on the Uncertainty of the Final Outcome (Read-Across for Data Gap Filling for Repeat Dose Toxicity)
Qualitative impact of metrics of chemical similarity on uncertainty in read-across for data gap filling (for repeat dose toxicity)	Low	Low	Low impact
Qualitative impact of chemical class on uncertainty in read-across for data gap filling (for repeat dose toxicity)	High	High	High impact
Qualitative impact of molecular similarity relating to toxicodynamics on uncertainty in read-across for data gap filling (for repeat dose toxicity)	High	For a specific mode of action, high; where no specific mode, low	Where a specific mode is present, high impact; otherwise - no impact
Qualitative impact of molecular similarity relating to toxicokinetics on uncertainty in read-across for data gap filling (for repeat dose toxicity)	High	High	High impact
Qualitative impact of molecular similarity relating to the formation of common metabolites or degradants on uncertainty in read-across for data gap filling (for repeat dose toxicity)	High	Where relevant, high; where not applicable, no impact (and need not be assessed)	When metabolism is relevant to (sub-)chronic toxicity – high impact, otherwise – no impact
Qualitative impact of chemical properties relating to toxicokinetics on uncertainty in read-across for data gap filling (for repeat dose toxicity)	High	High	High impact
Qualitative impact of toxicological data quality chemical properties on uncertainty in read-across for data gap filling (for repeat dose toxicity)	High	High	High impact

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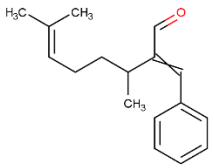
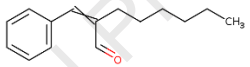
3.4 Examples of applying the proposed scheme to assessing uncertainties of chemical pairings

3.4.1 Assessment of overall uncertainty for a read-across to fill a data gap for skin sensitisation

2-Benzylidene-3,7-dimethyloct-6-enal lacks an EU REACH dossier, and no data on skin sensitisation were found. Therefore, it was used as the target for this illustration of uncertainty assessments of read-across predictions for skin sensitisation. It is a $C_{17}H_{22}O$ analogue, specifically a benzylidene with an aldehyde group attached to the alpha-carbon of the benzyl-alkene and an unsaturated branched aliphatic substituent on the beta-carbon of the benzyl-alkene. Its mechanism of sensitisation involves a benzylidene Michael addition (Enoch et al., 2008; 2011). Using 2D structure analysis to classify potential substances for read-across sources initially, the focus was on the benzylidene-substituted aldehydes with carbon chains ranging from C15 to C20. Literature searches identified only two compounds, 2-benzylideneoctanal and 3,3-diphenylprop-2-enal, with relevant *in vivo* data (i.e., local lymph node assay (LLNA) concentration required for a three-fold increase in lymph node cell proliferation compared with vehicle control (EC3) values). A subsequent search for smaller benzylidene-substituted aldehydes that could cause skin sensitisation revealed several compounds in the C9 to C13 range with reliable data; however, they were not evaluated as they were less similar to the analogues chosen.

The uncertainty schemes for using 2-benzylideneoctanal and 3,3-diphenylprop-2-enal as source substances are presented in Tables 3 and 4, respectively. Tables 3 and 4 draw upon and interpret information that would normally be captured in the data matrix to support read-across. More detailed descriptions of the mechanism of action of the target substance, 2-benzylidene-3,7-dimethyloct-6-enal, and a summary of the mechanisms of action and EC3 potency of various compounds that are relevant to it are reported in Supplementary Information 2.

466 Table 3. Uncertainty analysis of the read-across of skin sensitisation from 2-benzylideneoctanal to 2-benzylidene-3,7-dimethyloct-6-enal.

Data gap to be filled: skin sensitisation	2D structure	Key Properties Relating to Uncertainty
Target molecule: 2-benzylidene-3,7-dimethyloct-6-enal CAS # 84041-79-2 SMILES notation: CC(CCC=C(C)C)/C(=C\C1=CC=CC=C1)/C=O https://pubchem.ncbi.nlm.nih.gov/compound/6365928	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID401267459	Molecular Weight (MW): 242.4 Da* Log P (estimated): 5.18* Skin Permeability: logarithm of the permeability coefficient (log Kp) [log(cm/h)] = -0.677**
Source molecule: 2-benzylideneoctanal CAS # 101-86-0 SMILES notation: CCCCCC/C(=C\C1=CC=CC=C1)/C=O https://pubchem.ncbi.nlm.nih.gov/compound/1550884	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID4026684	Molecular Weight (MW): 216.3 Da* Log P (estimated): 4.68* Skin Permeability: log Kp [log(cm/h)]: -0.699**

Type and overall impact of uncertainty (refer to Table 2a)		Associated uncertainty to the read-across				
Type of uncertainty	**Impact on overall uncertainty of the type of uncertainty**	**Very low**	**Low**	**Moderate**	**High**	**Notes**
Metrics of chemical similarity	Low impact		**X**			Maximum Common Substructure (MCS Tanimoto): 0.63 (http://chemmine.ucr.edu/similarity/)
Chemical class	Low impact	**X**				Similar molecular formula and the same functional groups. Both aldehyde-substituted benzylidene with a β-alkyl group
Molecular similarity relating to toxicodynamics	High impact	**X**				Both have the same functional group α,β-unsaturated carbonyl, and no other functional group relevant to reactivity. As such, both are capable of acting by the same mechanisms of action (Michael addition and Schiff base formation)*** Rate of reactivity is similar, being moderately reactive with GSH****

Molecular similarity relating to toxicokinetics	Where relevant, low impact	**X**				Functional groups associated with highly similar absorption and distribution within the skin, in addition to the same metabolic pathways and elimination routes
Molecular similarity relating to the formation of common metabolites or degradants	Where relevant, high impact	**X**				Both direct-acting electrophiles have the same metabolic pathways
Chemical properties relating to toxicokinetics	Moderate impact	**X**				Highly similar ADME parameters, particularly with regard to skin absorption. Highly similar log P, MW, etc.
Source data quality	Moderate impact	**X**				Multi-replicates of LLNA following OECD TG 429
Overall uncertainty	The overall uncertainty is very low given the structural similarity between the two molecules. The highest-impact uncertainty for these analogues regarding skin sensitisation is the reactive mechanism of action. Both molecules have identical functional groups relevant to reactivity and similar reactivity rates, as identified by the *in silico* profilers; therefore, very low uncertainty is justified. If required, further experimental data (e.g., *in chemico* NAMs) could support this. The impact of metabolism/degradation for these compounds is low, as they are direct-acting. The two molecules are very similar in terms of physicochemical properties, particularly those affecting skin sensitisation, and meet the criteria stated in Table S1 for very low uncertainty. There is a negligible difference in predicted skin permeability. Low uncertainty in the chemical similarity metric does not significantly affect the overall uncertainty, as it has minimal impact.					

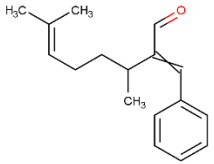
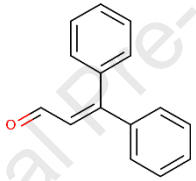
*Data from US EPA CompTox Dashboard (link under structure)

**Values from VEGA model: Skin Permeation (LogKp) model (Potts and Guy) 1.0.1

***OECD QSAR Toolbox (ver 4.8) Protein binding by OASIS

****OECD QSAR Toolbox (ver 4.8) Protein binding potency GSH

473 Table 4. Uncertainty analysis of the read-across of skin sensitisation from 3,3-diphenylprop-2-enal to 2-benzylidene-3,7-dimethyloct-6-enal.

Data gap to be filled: skin sensitisation	2D structure	Key Properties Relating to Uncertainty
Target molecule: 2-benzylidene-3,7-dimethyloct-6-enal CAS # 84041-79-2 SMILES notation: CC(CCC=C(C)C)/C(=C\C1=CC=CC=C1)/C=O https://pubchem.ncbi.nlm.nih.gov/compound/6365928	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID401267459	Molecular Weight (MW): 242.4 Da* Log P (estimated): 5.18* Skin Permeability: logarithm of the permeability coefficient (lo Kp) [log(cm/h)] = -0.677**
Source molecule: 3,3-diphenylprop-2-enal CAS # 1210-39-5 SMILES notation: C1=CC=C(C=C1)C(=CC=O)C2=CC=CC=C2 https://pubchem.ncbi.nlm.nih.gov/compound/71027	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID2049210	Molecular Weight (MW): 208.26* Log P (estimated): 3.15* Skin Permeability: log Kp [log(cm/h)]: -1.536**

Type and overall impact of uncertainty (refer to Table 2a)		Associated uncertainty to the read-across				
Type of uncertainty	**Impact on overall uncertainty**	**Very low**	**Low**	**Moderate**	**High**	**Notes**
Metrics of chemical similarity	Low impact			**X**		Maximum Common Substructure (MCS Tanimoto): 0.46 (http://chemmine.ucr.edu/similarity/)
Chemical class	Low impact		**X**			Similar molecular formula and the same functional groups; however, the source molecule has two phenyl rings. Both aldehyde-substituted benzylidene with a β-alkyl group
Molecular similarity relating to toxicodynamics	High impact			**X**		Both have the same functional group α,β-unsaturated carbonyl and no other functional group relevant to reactivity. As such, both are capable of acting by the same mechanisms of action (Schiff base formation) although the target molecule may also act as a Michael acceptor*** Rate of reactivity is similar being moderately reactive with GSH****

Molecular similarity relating to toxicokinetics	Where relevant, low impact			**X**		Differences in functional groups and molecular scaffolds may be associated with different absorption and distribution within the skin. In addition, there may be differences in metabolic pathways and elimination routes
Molecular similarity relating to the formation of common metabolites or degradants	Where relevant, high impact	**X**				Both direct-acting electrophiles have the same metabolic pathways
Chemical properties relating to toxicokinetics	Moderate impact			**X**		Similar ADME parameters, with regard to skin absorption, the permeability of the source molecule is 1 log unit lower than the target. Highly similar MW, but a significant difference in log P (2 log units).
Source data quality	Moderate impact		**X**			Multi-replicates of LLNA following OECD TG 429
Overall uncertainty	The overall uncertainty is moderate, based on the structural similarity between the two molecules. The highest-impact uncertainty for these analogues regarding skin sensitisation is the reactive mechanism of action, as the source molecule was not identified as a Michael acceptor. Both molecules are predicted to have similar reactivity rates, as determined by the *in silico* profiler. If required, further experimental data (e.g., *in chemico* NAMs) could support this. The impact of metabolism/degradation for these compounds is very low, as they are direct-acting. The two molecules are similar in terms of physicochemical properties, particularly those affecting skin sensitisation, with moderate uncertainty. This is attributed to a significant difference in log P (the source has a lower log P) and lower skin permeability.					

*Data from US EPA CompTox Dashboard (link under structure)

**Values from VEGA model: Skin Permeation (LogKp) model (Potts and Guy) 1.0.1

***OECD QSAR Toolbox (ver 4.8) Protein binding by OASIS

****OECD QSAR Toolbox (ver 4.8) Protein binding potency GSH

Based on the results shown in Tables 3 and 4, 2-benzylideneoctanal is the more suitable source compound for filling the data gap in skin sensitisation for 2-benzylidene-3,7-dimethyloct-6-enal, as it has the lower overall uncertainty. LLNA data from lower molecular weight benzylidene-substituted aldehydes (see Supplementary Information 2) add weight-of-evidence to the read-across evaluated in Table 3.

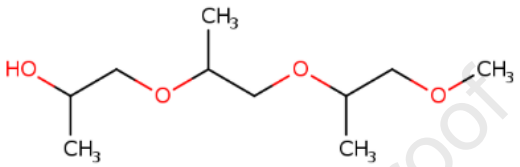
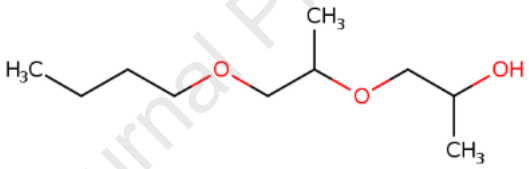
As detailed in Supplementary Information 2, aldehydes with similar hydrocarbon scaffolds, but without a carbon-to-carbon double bond or a non-conjugated carbon-to-carbon double bond (i.e., non-benzylidenealkanals), are either non-sensitisers or sensitisers through a reactive mechanism other than Michael addition.

3.4.2 Assessment of overall uncertainty for a read-across to fill a data gap for repeated dose toxicity

1-[1-(1-Methoxypropan-2-yloxy)propan-2-yloxy]propan-2-ol or tripropyleneglycol monomethyl ether has no REACH dossier, and no repeated dose toxicity data were found. Therefore, it was taken as the target for this illustration of assessing uncertainties read-across. It is a $C_{10}H_{22}O_4$ analogue, which is a secondary alcohol with a branched saturated aliphatic scaffold containing three ether linkages, including a terminal methoxy group. Searches of the scientific literature revealed several potential read-across source substances.

Explanations of the metabolic rationale for eliminating primary and tertiary alcohols but including secondary alcohols and corresponding ketones in searching for source substances for the target 1-[1-(1-methoxypropan-2-yloxy)propan-2-yloxy]propan-2-ol and relevant rodent sub-chronic repeat dose toxicity data are reported in Supplementary Information 3. Using the uncertainty assessment scheme described above, four of these substances were evaluated in Tables 5 to 8. Tables 5 to 8 draw upon and interpret information that would normally be captured in the data matrix to support read-across.

502 Table 5. Uncertainty analysis of the read-across of sub-chronic repeated dose toxicity from 1-(1-butoxypropan-2-yloxy)propan-2-ol to 1-[2-(2-methoxy-1-
503 methylethoxy)-1-methylethoxy]propan-2-ol.

Data gap to be filled: sub-chronic repeated dose toxicity	2D structure	Key Properties Relating to Uncertainty
Target molecule: 1-[2-(2-methoxy-1-methylethoxy)-1-methylethoxy]propan-2-ol CAS # 20324-33-8 SMILES notation: CC(COC(C)COC(C)COC)O https://pubchem.ncbi.nlm.nih.gov/compound/30111	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID6021616	Molecular Weight (MW): 206.3 Da* Log P (estimated): 0.38* Elimination half-life: 3.4 hours**
Source molecule: 1-(1-butoxypropan-2-yloxy)propan-2-ol CAS # 29911-28-2 SMILES notation: CCCCOCC(C)OCC(C)O https://pubchem.ncbi.nlm.nih.gov/compound/24752	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID8027959	Molecular Weight (MW): 190.3 Da* Log P (estimated): 1.42* Elimination half-life: 3.2 hours**

Type and overall impact of uncertainty (refer to Table 2b)		Associated uncertainty to the read-across				
Type of uncertainty	**Impact on overall uncertainty**	**Very low**	**Low**	**Moderate**	**High**	**Notes**
Metrics of chemical similarity	Low impact		X			Maximum Common Substructure (MCS Tanimoto): 0.69 (http://chemmine.ucr.edu/similarity/)
Chemical class	High impact	X				Same, polyether-substituted aliphatic secondary alcohol
Molecular similarity relating to toxicodynamics	Low impact	X				Similar potential reactive centres: secondary alcohol and alkoxy groups Both target and source contain the alert for "Propylene Glycol Ethers Category (Less susceptible) No Rank" which includes four structurally related propylene glycol ethers or the acetates.***

Molecular similarity relating to toxicokinetics	High impact	**X**				Similar absorption and distribution, and the same metabolic pathways and elimination routes
Molecular similarity relating to the formation of common metabolites or degradants	High impact	**X**				Same, oxidation to the corresponding ketone, hydroxylation, and phase II glucuronidation
Chemical properties relating to toxicokinetics	High impact	**X**				Target has a similar MW, but a higher log P by approximately 1 log unit. Rate of clearance is predicted to be comparable. Very low uncertainty is assigned based on the rate of clearance.
Source data quality	High impact	**X**				GLP-compliant, appropriate OECD test guidelines, multiple exposure schemes
Overall uncertainty	The overall uncertainty is very low due to structural similarity and identical or highly similar ADME properties. The data quality from read-across is exceptional, with both NOAEL and LOAEL values for the three routes of exposure. On this occasion, the approximately 1 log-unit difference in log P was deemed not to influence toxicity, as clearance rates are expected to be similar. Low uncertainty in the chemical similarity metric does not affect the overall uncertainty, as it has low impact.					

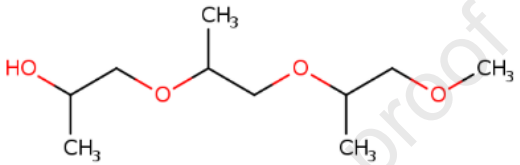
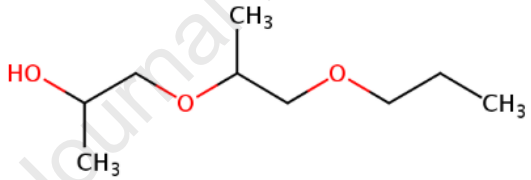
*Data from US EPA CompTox Dashboard (link under structure)

**Values from VEGA model: Total body elimination half-life (QSARINS) 1.0.1

***OECD QSAR Toolbox (ver 4.8) Repeated dose (HESS) profiler

508 Table 6. Uncertainty analysis of the read-across of sub-chronic repeated dose toxicity from 1-(1-propoxypropan-2-yloxy)propan-2-ol to 1-[2-(2-methoxy-1-
509 methylethoxy)-1-methylethoxy]propan-2-ol.

510

Data gap to be filled: sub-chronic repeated dose toxicity	2D structure	Key Properties Relating to Uncertainty
Target molecule: 1-[2-(2-methoxy-1-methylethoxy)-1-methylethoxy]propan-2-ol CAS # 20324-33-8 SMILES notation: CC(COC(C)COC(C)COC)O https://pubchem.ncbi.nlm.nih.gov/compound/30111	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID6021616	Molecular Weight (MW): 206.3 Da* Log P (estimated): 0.38* Elimination half-life: 3.4 hours**
Source molecule: 1-(1-propoxypropan-2-yloxy)propan-2-ol CAS # 29911-27-1 SMILES notation: CCCOCC(C)OCC(C)O https://pubchem.ncbi.nlm.nih.gov/compound/121752	 Structure from: https://comptox.epa.gov/dashboard/chemical/properties/DTXSID3033276	Molecular Weight (MW): 176.3 Da* Log P (estimated): 0.97* Elimination half-life: 3.1 hours**

Type and overall impact of uncertainty (refer to Table 2b)		Associated uncertainty to the read-across				
Type of uncertainty	**Impact on overall uncertainty**	**Very low**	**Low**	**Moderate**	**High**	**Notes**
Metrics of chemical similarity	Low impact	X				Maximum Common Substructure (MCS Tanimoto): 0.73 (http://chemmine.ucr.edu/similarity/)
Chemical class	High impact	X				Same, polyether-substituted aliphatic secondary alcohol
Molecular similarity relating to toxicodynamics	Low impact	X				Similar potential reactive centres: secondary alcohol and alkoxy groups

						Both target and source contain the alert for "Propylene Glycol Ethers Category (Less susceptible) No Rank" which includes four structurally related propylene glycol ethers or the acetates.***
Molecular similarity relating to toxicokinetics	High impact	**X**				Similar absorption and distribution, and the same metabolic pathways and elimination routes
Molecular similarity relating to the formation of common metabolites or degradants	High impact	**X**				Same, oxidation to the corresponding ketone, hydroxylation, and phase II glucuronidation
Chemical properties relating to toxicokinetics	High impact	**X**				Similar log P, MW, etc., reflecting the minor differences in the number of C- and O-atoms. Rate of clearance is very similar.
Source data quality	High impact		**X**			GLP-compliant, most appropriate OECD test guidelines, no LOAEL
Overall uncertainty	The overall uncertainty is low based on the uncertainty in the toxicological data, i.e., no reported LOAEL. The ADME and chemical properties are very similar, as are the toxicodynamics.					

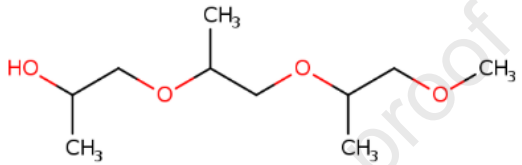
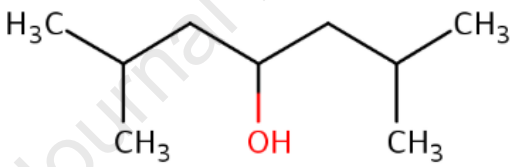
*Data from US EPA CompTox Dashboard (link under structure)

**Values from VEGA model: Total body elimination half-life (QSARINS) 1.0.1

***OECD QSAR Toolbox (ver 4.8) Repeated dose (HESS) profiler

515 Table 7. Uncertainty analysis of the read-across of sub-chronic repeated dose toxicity from 2,6-dimethylheptan-4-ol to 1-[2-(2-methoxy-1-methylethoxy)-1-
516 methylethoxy]propan-2-ol.

517

Data gap to be filled: sub-chronic repeated dose toxicity	2D structure	Key Properties Relating to Uncertainty
Target molecule: 1-[2-(2-methoxy-1-methylethoxy)-1-methylethoxy]propan-2-ol CAS # 20324-33-8 SMILES notation: CC(COC(C)COC(C)COC)O https://pubchem.ncbi.nlm.nih.gov/compound/30111	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID6021616	Molecular Weight (MW): 206.3 Da* Log P (estimated): 0.38* Elimination half-life: 3.4 hours**
Source molecule: 2,6-dimethylheptan-4-ol CAS # 108-82-7 SMILES notation: CC(C)CC(CC(C)C)O https://pubchem.ncbi.nlm.nih.gov/compound/7957	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID8026802	Molecular Weight (MW): 144.3 Da* Log P (estimated): 3.15* Elimination half-life: 5.6 hours**

Type and overall impact of uncertainty (refer to Table 2b)		Associated uncertainty to the read-across				
Type of uncertainty	**Impact on overall uncertainty**	**Very low**	**Low**	**Moderate**	**High**	**Notes**
Metrics of chemical similarity	Low impact			**X**		Maximum Common Substructure (MCS Tanimoto): 0.20 (http://chemmine.ucr.edu/similarity/)
Chemical class	High impact			**X**		Similar aliphatic secondary alcohols but lacking ether linkages
Molecular similarity relating to toxicodynamics	Low impact			**X**		Similar potential reactive centre: secondary alcohol, but missing the alkoxy groups The target contains the alert for "Propylene Glycol Ethers Category (Less susceptible) No Rank" which includes four structurally related

						propylene glycol ethers or the acetates. However, this is lacking from the source molecule***
Molecular similarity relating to toxicokinetics	High impact		**X**			Similar absorption and distribution, and the same metabolic pathways and elimination routes
Molecular similarity relating to the formation of common metabolites or degradants	High impact	**X**				Same, oxidation to the corresponding ketone, hydroxylation, and phase II glucuronidation
Chemical properties relating to toxicokinetics	High impact		**X**			The source molecule has a greater log P, but is slower to be eliminated, i.e., will be more bioavailable. The slower elimination mitigates, to some extent, the large difference in log P.
Source data quality	High impact		**X**			GLP-compliant, less appropriate OECD test guidelines; test of a 70/30 binary mixture; NOAEL reported with a safety factor of 3
Overall uncertainty	The overall uncertainty is moderate as the structural differences between the target and source analogue are significant. The ADME and chemical properties reflect the differences in the number of carbon atoms and the number of alkoxy groups. The quality of the data being read across is severely diminished by the test being conducted in TG 422, where the test material is a binary mixture with 70% target chemical and does not attain a LOAEL value.					

518 *Data from US EPA CompTox Dashboard (link under structure)

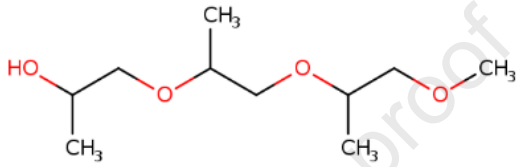
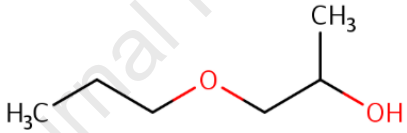
519 **Values from VEGA model: Total body elimination half-life (QSARINS) 1.0.1

520 ***OECD QSAR Toolbox (ver 4.8) Repeated dose (HESS) profiler

521

522 Table 8. Uncertainty analysis of the read-across of sub-chronic repeated dose toxicity from 1-propoxypropan-2-ol to 1-[2-(2-methoxy-1-methylethoxy)-1-
523 methylethoxy]propan-2-ol.

524

Data gap to be filled: sub-chronic repeated dose toxicity	2D structure	Key Properties Relating to Uncertainty
Target molecule: 1-[2-(2-methoxy-1-methylethoxy)-1-methylethoxy]propan-2-ol CAS # 20324-33-8 SMILES notation: CC(COC(C)COC(C)COC)O https://pubchem.ncbi.nlm.nih.gov/compound/30111	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID6021616	Molecular Weight (MW): 206.3 Da (1) Log P (estimated): 0.38 (1) Elimination half-life: 3.4 hours (2) (1) Data from US EPA CompTox Dashboard (link under structur (2) Values from VEGA model: Total body elimination half-life (QSARINS) 1.0.1
Source molecule: 1-propoxypropan-2-ol CAS # 1569-01-3 SMILES notation: CCCOCC(C)O https://pubchem.ncbi.nlm.nih.gov/compound/15286	 Structure from: https://comptox.epa.gov/dashboard/chemical/details/DTXSID5029217	Molecular Weight (MW): 118.2 Da (1) Log P (estimated): 0.60 (1) Elimination half-life: 2.6 hours (2) (1) Data from US EPA CompTox Dashboard (link under structur (2) Values from VEGA model: Total body elimination half-life (QSARINS) 1.0.1

Type and overall impact of uncertainty (refer to Table 2b)		Associated uncertainty to the read-across				
Type of uncertainty	**Impact on overall uncertainty**	**Very low**	**Low**	**Moderate**	**High**	**Notes**
Metrics of chemical similarity	Low impact		X			Maximum Common Substructure (MCS Tanimoto): 0.47 (http://chemmine.ucr.edu/similarity/)
Chemical class	High impact		X			Similar aliphatic secondary alcohol and ether linkage
Molecular similarity relating to toxicodynamics	Low impact			X		Similar potential reactive centre: secondary alcohol and a single alkoxy group The target contains the alert for "Propylene Glycol Ethers Category (Less susceptible) No Rank" which includes four structurally related propylene glycol ethers or the acetates. However, this is lacking from the source molecule (3)

						(3) OECD QSAR Toolbox (ver 4.8) Repeated dose (HESS) profiler
Molecular similarity relating to toxicokinetics	High impact			**X**		Dissimilar absorption and distribution, but the same metabolic pathways and elimination routes
Molecular similarity relating to the formation of common metabolites or degradants	High impact	**X**				Same, oxidation to the corresponding ketone, hydroxylation, and phase II glucuronidation
Chemical properties relating to toxicokinetics	High impact			**X**		Similar log P, but the source has significantly lower MW and faster predicted clearance.
Source data quality	High impact			**X**		GLP-compliant, less appropriate route of exposure, NOAEC highest concentration tested, no LOAEC
Overall uncertainty	The overall uncertainty is moderate due to the structural differences between the target and source analogue, which lead to significant differences in clearance. The ADME and chemical properties reflect differences in the number of carbon atoms and alkoxy groups. The quality of the data being read across is significantly reduced because the NOAEC is the highest concentration tested, and there is no LOAEC value.					

525

Based on the results shown in Tables 5-8, 1-(1-butoxypropan-2-yloxy)propan-2-ol is the most suitable source compound for filling the data gap in subchronic repeated dose toxicity for 1-[1-(1-methoxypropan-2-yloxy)propan-2-yloxy]propan-2-ol, as it has the lowest overall uncertainty. The second-best source chemical, 1-(1-propoxypropan-2-yloxy)propan-2-ol, provides additional *in vivo* evidence supporting the read-across based on the best chemical pairings. 2,6-Dimethylheptan-4-ol and 1-propoxypropan-2-ol are both less ideal source materials, having moderate overall uncertainty due to significant and quantifiable structural differences.

3.5 Analysis of uncertainty in published read-across predictions to identify tolerable uncertainty

RIFM's published safety assessments of fragrance materials were examined. Most contained at least one example of read-across to fill a data gap for human health and, occasionally, environmental endpoints. Of the published safety assessments, 25 were chosen for assessment of the uncertainties in the read-across predictions according to the criteria stated in Section 3.2 for skin sensitisation and repeated dose toxicity. The description of the read-across process, along with a summary of the read-across approaches, is summarised in Supplementary Information 4 with the outcomes of the uncertainty assessment in Tables S4.1 and S4.2.

These fragrance material safety assessments represented different chemical classes within the fragrance inventory (Date et al., 2020). The endpoint distributions of read-across reflect the distribution of endpoints across over 1,500 published assessments containing one or more read-across justifications (Moustakas et al., 2022) – although only two endpoints for a small number of examples were assessed here. Aside from one judgment, which was found to be "moderate" for repeated dose toxicity, the authors considered the overall uncertainty of the read-across pairings to be "very low" or "low". It is acknowledged that fragrance ingredients are an exceptionally data-rich segment of the chemical universe, with limited structural diversity, and that these factors contribute to the overall uncertainties being so low. The findings support the idea that tolerable uncertainty is likely ensured if the overall judgment indicates low or very low uncertainty.

4 Discussion

Read-across is one of the most important, if not the most widely used, *in silico* NAMs. Although their acceptance rates vary, they are progressively improving (Hartung and Rovida 2025). Several read-

558 across scenarios have been identified (Patlewicz et al., 2025). The most common use is filling data
559 gaps, especially for replacing animal tests in risk assessments for industrial and regulatory purposes.
560 Generally, using read-across to support risk assessment involves some of the strictest acceptance
561 criteria, with acceptability and confidence levels, along with supporting information, being
562 determined by industrial standards or regulatory requirements. One way to evaluate read-across is to
563 develop methods for identifying and characterising uncertainty. The recent EFSA guidance reinforces
564 the idea of "tolerable uncertainty," but it does not specify the acceptable levels. This study aimed to
565 develop and utilise a novel approach to assess qualitatively the main aspects of uncertainty in read-
566 across based, in part, on EFSA's guidance relating to uncertainty and read-across (EFSA Scientific
567 Committee, 2018b; 2025).

568

569 4.1 Qualitative assessment of the uncertainty in read-across

570 This study identified seven core elements that contribute to uncertainty in read-across. The goal was
571 to simplify the process of identifying uncertainty in read-across into a clear, manageable set of criteria.

572 A variety of approaches to characterise and quantify uncertainty in *in silico* toxicology have been
573 previously explored and influenced this work. These include methods such as Bayesian learning (Allen
574 et al., 2022) and conformal predictions (Sapounidou et al., 2023), among others (Sahlin et al., 2011).
575 The approach taken in this study was not based on statistical methods but aimed to develop a
576 transparent, flexible, and easily applicable scheme that could be used to evaluate read-across and
577 demonstrate tolerable uncertainty. This study employed four levels of uncertainty, based on principles
578 outlined by the EFSA Scientific Committee (2018a, b). The four ordinal classifications of uncertainty
579 rely on expert analysis and enough information to assess the acceptability of the read-across. The
580 primary uncertainty levels are "very low" and "low," and it is expected that these would be acceptable
581 for most purposes. However, only very low levels of uncertainty may be inevitably acceptable for
582 certain uses, such as filling data gaps for regulatory-related risk assessments. Moderate levels of
583 uncertainty may be tolerable in certain circumstances, and when the impact on the overall outcome
584 is low. High levels of uncertainty are, in most cases, unlikely to be tolerable; the high uncertainty
585 grading is broad (note that no very high classification is proposed) and intended to capture any level
586 of non-tolerable uncertainty. It is unlikely for a read-across prediction with significantly high levels of
587 uncertainty to ever advance beyond the formative phase unless it is mitigated by low, or no, impact
588 or until further data and / or information is included to create a weight-of-evidence (Escher et al.,
589 2019; 2022).

To meet current regulatory standards and criteria, classifying uncertainty in binary terms (e.g., acceptable/unacceptable) is of little, or no, value. Ordinal classifications (e.g., classification with three or more categories) have been proposed. Schultz et al. (2015) proposed three levels: high, moderate, and low. ECHA RAAF has five AOs (ECHA, 2017). These can be broadly mapped onto the scheme proposed in this study, as demonstrated in Table 1. It is intended that every aspect of the read-across uncertainty scheme be flexible and adaptable. If there is a need to adjust the definitions or impact of the relative levels of uncertainty, this would be possible. However, schemes with a high number of classifications (i.e., more than five) often struggle to achieve a majority, let alone a consensus, of expert opinions.

As illustrated in the examples (Tables 3-8), an overall uncertainty statement for the read-across can also be derived from the ordinal values in the proposed scheme. Three factors contribute to this statement: 1) the overall impact on the uncertainty of the final prediction, 2) the magnitude of uncertainty associated with the endpoint being filled, and 3) the sensitivity of uncertainty to the read-across concept. However, the contributions of these factors vary. Only one uncertainty criterion (chemical-similarity metrics) has an overall impact below the high level.

When the specified similarity is relevant to the read-across chemical pairing, the overall impact on the final prediction's uncertainty and the magnitude of uncertainty associated with the endpoint being filled are typically highly correlated. Therefore, overall uncertainty is usually driven by the high-impact uncertainties. The general consideration is that overall uncertainty cannot be lower than the highest level of uncertainty among the “high impact” uncertainties. Overall uncertainty can be lower than the level of uncertainty associated with “moderate/low impact" uncertainties. However, concrete examples of such occurrences are not available as failed read-across predictions are not published.

Tolerable uncertainty would usually be established during the problem formulation stage of read-across (EFSA Scientific Committee, 2025) and will be specific to the context and use. A key aim of this study and a notable feature of the proposed approach is to identify and define tolerable uncertainty. This method would enable the expression of levels of tolerable uncertainty, and, by applying the seven criteria in a standard format, can vastly improve their application. Applying the reported uncertainty assessment scheme to the 25 published read-across assessments for skin sensitisation (see Table S4.1) and repeated dose toxicity (see Table S4.2), we obtained only one prediction that has an overall uncertainty greater than “low”. The overall uncertainty of moderate is explainable, and could be improved by the addition of further data to reduce uncertainty.

A key feature of the proposed uncertainty assessment scheme is its flexibility. Although only skin sensitisation and subchronic repeat dose systemic toxicity are demonstrated, it can be adapted to

other endpoints. Typically, the scheme details for skin sensitisation can be easily modified for mutagenesis, clastogenesis, and likely photo irritation. Similarly, the scheme details for repeat dose toxicity should be adjustable to include fertility and developmental toxicity.

4.2 Regulatory relevance of the proposed scheme and template for the assessment of uncertainty in read-across.

The scheme presented provides for the pragmatic assessment of overall uncertainty in a flexible manner and the opportunity for tolerable uncertainty to be stated as part of the regulatory framework. Specifically with regard to ECHA RAAF (ECHA, 2017), the above examples are directly relatable to ECHA RAAF Scenario 2 (analogue approach for which the read-across hypothesis is based on different compounds with qualitatively similar properties). With regard to the AEs in RAAF Scenario 2, the approach to assess uncertainty will directly support:

- AE A.2 Link of structural similarities and differences with the proposed prediction

In addition, it will provide indirect support to assess:

- AE 2.2 Common underlying mechanism, qualitative aspects
- AE 2.3 Common underlying mechanism, quantitative aspects

The scheme will need to be adapted for each endpoint assessed, particularly with a better understanding of the impact of each of the uncertainty criteria. It is envisioned that the requirements to provide evidence to meet the information requirements within current, and future, chemicals' legislation will dictate that overall uncertainty must be preferentially "very low" as described in this approach, or occasionally "low" with reasonable justification. These uncertainties have not previously been placed in a defined, and quantifiable context as is provided in this approach. The scheme and template also allow for the inclusion of further evidence to support the structural characterisation, such as the inclusion of NAM data.

The scheme can also be adapted to meet the needs of other RAAF scenarios (not detailed herein). In addition, it can be used for other regulatory purposes, where similarity criteria may be more relaxed. An example is the recent revision of the EU Classification, Labelling and Packaging (CLP) (EC, 2024), where greater emphasis has been placed on the use of groups for harmonised classifications (CLH). CLH may allow for more relaxed consideration of similarity, for instance, putting emphasis on similarity in mode of action, rather than 2D structure. Such relaxation of criteria can be accommodated in the scheme in two ways. One possibility is that the relative impact of the uncertainty criteria could be reduced, emphasising the structural basis of the mode of action. Alternatively, in the flexible

application of the scheme, higher uncertainty in the less relevant criteria (e.g., for structural similarity) could be tolerable on an *ad hoc* and well-justified, basis. These latter points could be made clear in the overall problem formulation.

5. Summary

It has been over a decade since Ball and others broached the question, "How much uncertainty in read-across predictions is too much?" (Ball et al., 2014). Some of the earlier findings remain valid today. The differences in establishing tolerable uncertainty depend on the data gap being filled, specifically the context (regulatory or otherwise) and endpoint. Data gap ranging from observations at the molecular or cellular level to those at the organismal level. Some data gaps exist for well-studied and well-understood endpoints, while others pertain to less well-understood endpoints. Additionally, some data gaps are expressed in binary ordinal terms (toxicity or non-toxicity), while others are quantified as continuous potency. These factors impact the uncertainties associated with accepting a read-across prediction. Specific policies and/or regulations will also influence tolerable uncertainty, such as assessing every substance in an inventory or avoiding animal testing. While determining tolerable uncertainty in read-across remains expert-derived and determined on a case-by-case basis, the criteria for evaluating and standards for quantifying uncertainty have become more established.

The scheme for assessing uncertainty in read-across proposed in this study is intended to be flexible and adaptable. It defines fully the levels of uncertainty and their relative impact. While different degrees of structural similarity and various data arrays are observed in published read-across predictions, the uncertainties associated with these predictions can be classified into two situations. The read-across is directly actionable based on data from a source chemical that strictly or near-strictly meets the structural definition of the target substance. However, as the structural definition of the chemical grouping becomes more lenient, uncertainty tends to increase, making the read-across not actionable without considering additional forms of chemical similarity. The approach presented herein is intended to assist in translating the concept of uncertainties in read-across into a scheme that recognises, evaluates, and details relevant and overall uncertainties.

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Disclaimer

This work reflects only the authors' views, and the European Commission is not responsible for any use that may be made of the information it contains.

Conflict of Interest

MTDC is a current member, and TWS is a former member, of the Expert Panel for Fragrance Safety (https://fragrancesafetypanel.org).

CRediT authorship contribution statement

Mark T.D. Cronin: Conceptualization; Writing – review and editing; **Terry W. Schultz:** Conceptualization; Writing – original draft.

Statement of the Use of AI

The AI Tool "ChatGPT (GPT-5)" (OpenAI, 2025. *ChatGPT*, https://chat.openai.com/) was used for the initial formatting of references to a standardised format in the main manuscript (not the Supplementary Information). The authors manually checked and finalised the presentation of each reference and verified the veracity of every citation.

References

Alépée N, Tourneix F, Singh A, Ade N, Grégoire S. (2023) Off to a good start? Review of the predictivity of reactivity methods modelling the molecular initiating event of skin sensitization. *ALTEX* 40: 606-618. doi: 10.14573/altex.2212201

Allen THE, Middleton AM, Goodman JM, Russell PJ, Kukic, P (2022) Towards quantifying the uncertainty in in silico predictions using Bayesian learning. *Comp. Toxicol.* 23: 100228. doi: 10.1016/j.comtox.2022.100228

Api AM, Belsito D, Bruze M, Cadby P, Calow P, Dagli ML, Dekant W, Ellis G, Fryer AD, Fukayama M, Griem P, Hickey C, Kromidas L, Lalko JF, Liebler DC, Miyachi Y, Politano VT, Renskers K, Ritacco G, Salvito D, Schultz TW, Sipes IG, Smith B, Vitale D, Wilcox DK (2015) Criteria for the Research Institute

724 for Fragrance Materials, Inc. (RIFM) safety evaluation process for fragrance ingredients. *Food Chem.*
725 *Toxicol*. 82 Suppl: S1-S19. doi: 10.1016/j.fct.2014.11.014

726 Bajusz D, Rácz A, Héberger K (2015) Why is Tanimoto index an appropriate choice for fingerprint-based
727 similarity calculations? *J. Cheminform*. 7: 20. doi: 10.1186/s13321-015-0069-3

728 Ball N, Bartels M, Budinsky R, Klapacz J, Hays S, Kirman C, Patlewicz G. (2014) The challenge of using
729 read-across within the EU REACH regulatory framework; how much uncertainty is too much?
730 Dipropylene glycol methyl ether acetate, an exemplary case study. *Regul. Toxicol. Pharmacol*. 68: 212-
731 221. doi: 10.1016/j.yrtph.2013.12.007

732 Barnett RE, Lawson TN, Rivetti C, Barata C, Cronin MTD, Lacorte S, Lloyd GR, Weber RJM, Smith MJ,
733 Southam AD, Biales A, Koehrn K, Campos B, Colbourne JK, Hodges G, Viant MR (2025) Substantiating
734 chemical groups for read-across using molecular response profiles. *Regul. Toxicol. Pharmacol*. 162:
735 105894. doi: 10.1016/j.yrtph.2025.105894.

736 Blackburn K, Stuard SB (2014) A framework to facilitate consistent characterization of read across
737 uncertainty. *Regul. Toxicol. Pharmacol*. 68: 353-62. doi: 10.1016/j.yrtph.2014.01.004

738 Boyer S, Arnby CH, Carlsson L, Smith J, Stein V, Glen RC (2007) Reaction site mapping of xenobiotic
739 biotransformations. *J. Chem. Inf. Model*. 47: 583-590. doi: 10.1021/ci600376q

740 Cereto-Massagué A, Ojeda MJ, Valls C, Mulero M, Garcia-Vallvé S, Pujadas G (2015) Molecular
741 fingerprint similarity search in virtual screening. *Methods* 71: 58-63. doi:
742 10.1016/j.ymeth.2014.08.005

743 Cronin MTD, Enoch SJ, Mellor CL, Przybylak KR, Richarz AN, Madden JC (2017) *In silico* prediction of
744 organ level toxicity: Linking chemistry to adverse effects. *Toxicol. Res*. 33: 173-182. doi:
745 10.5487/TR.2017.33.3.173

746 Date MS, O'Brien D, Botelho DJ, Schultz TW, Liebler DC, Penning TM, Salvito DT (2020) Clustering a
747 chemical inventory for safety assessment of fragrance ingredients: Identifying read-across analogs to
748 address data gaps. *Chem. Res. Toxicol*. 33: 1709-1718. doi: 10.1021/acs.chemrestox.9b00518

749 De Abrew KN, Natoli T, Lester CC, Wang X, Shobair M, Subramanian A, Daston GP (2022) A New
750 Approach Methodology (NAM) based assessment of butylated hydroxytoluene (BHT) for endocrine
751 disruption potential. *Toxicol. Sci*. 190: 227-241. doi: 10.1093/toxsci/kfac099

752 Dimitrov SD, Diderich R, Sobanski T, Pavlov TS, Chankov GV, Chapkanov AS, Karakolev YH, Temelkov
753 SG, Vasilev RA, Gerova KD, Kuseva CD, Todorova ND, Mehmed AM, Rasenberg M, Mekenyan OG
754 (2016) QSAR Toolbox - workflow and major functionalities. *SAR QSAR Environ. Res*.27: 203-219. doi:
755 10.1080/1062936X.2015.1136680

756 EC (2024) Regulation (EU) 2024/2865 of the European Parliament and of the Council of 23 October
757 2024 amending Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances
758 and mixtures (Text with EEA relevance). *Off. J. Eur. Union L,* 2024/2865, 20 November 2024. Available
759 from https://eur-lex.europa.eu/eli/reg/2024/2865/oj (accessed 17 September 2025).

760 ECHA (2008) *Guidance on Information Requirements and Chemical Safety Assessment. Chapter R.6:*
761 *QSARs and Grouping of Chemicals*. European Chemical Agency, Helsinki, Finland. Available from:
762 https://echa.europa.eu/documents/10162/17224/information_requirements_r6_en.pdf/77f49f81-
763 b76d-40ab-8513-4f3a533b6ac9?t=1322594777272 (accessed 27 August 2025).

764 ECHA (2017) *Read-Across Assessment Framework (RAAF)*. European Chemicals Agency, Helsinki,
765 Finland. ECHA-17-R-01-EN. doi: 10.2823/619212. Available
766 from https://data.europa.eu/doi/10.2823/619212

767 ECHA (2023) *The Use of Alternatives to Testing on Animals for the REACH Regulation. Fifth Report*
768 *under Article 117(3) of the REACH Regulation*. European Chemical Agency, Helsinki, Finland. ECHA-23-

R-07-EN. doi: 10.2823/805454. Available from: https://echa.europa.eu/documents/10162/23919267/230530_117_3_alternatives_test_animals_2023_en.pdf/9cfc291e-9baf-ffa2-466c-2bc2c6f06b8e?t=1685428213290

EFSA Scientific Committee; Benford D, Halldorsson T, Jeger MJ, Knutsen HK, More S, Naegeli H, Noteborn H, Ockleford C, Ricci A, Rychen G, Schlatter JR, Silano V, Solecki R, Turck D, Younes M, Craig P, Hart A, Von Goetz N, Koutsoumanis K, Mortensen A, Ossendorp B, Germini A, Martino L, Merten C, Mosbach-Schulz O, Smith A, Hardy A (2018a) The principles and methods behind EFSA's guidance on uncertainty analysis in scientific assessment. *EFSA J.* 16(1): e05122. doi: 10.2903/j.efsa.2018.5122

EFSA Scientific Committee; Benford D, Halldorsson T, Jeger MJ, Knutsen HK, More S, Naegeli H, Noteborn H, Ockleford C, Ricci A, Rychen G, Schlatter JR, Silano V, Solecki R, Turck D, Younes M, Craig P, Hart A, Von Goetz N, Koutsoumanis K, Mortensen A, Ossendorp B, Martino L, Merten C, Mosbach-Schulz O, Hardy A (2018b) Guidance on uncertainty analysis in scientific assessments. *EFSA J.* 16: e05123. doi: 10.2903/j.efsa.2018.5123

EFSA Scientific Committee; Bennekou SH, Allende A, Bearth A, Casacuberta J, Castle L, Coja T, Crépet A, Halldorsson T, Hoogenboom LR, Jokelainen P, Knutsen H, Koutsoumanis K, Lambré C, Nielsen S, Turck D, Civera AV, Villa RE, Zorn H, Benfenati E, Benigni R, Chaudhry Q, Farcal L, Kass G, Nathanail A, Paini A, Serafimova R (2025) Guidance on the use of read-across for chemical safety assessment in food and feed. *EFSA J*. 23: e9586. doi: 10.2903/j.efsa.2025.9586

Enoch SJ, Cronin MTD (2010) A review of the electrophilic reaction chemistry involved in covalent DNA binding. *Crit. Rev. Toxicol*. 40: 728-748. doi: 10.3109/10408444.2010.494175

Enoch SJ, Ellison CM, Schultz TW, Cronin MTD (2011) A review of the electrophilic reaction chemistry involved in covalent protein binding relevant to toxicity. *Crit. Rev. Toxicol*. 41: 783-802. doi: 10.3109/10408444.2011.598141

Enoch SJ, Roberts DW (2013) Predicting skin sensitization potency for Michael acceptors in the LLNA using quantum mechanics calculations. *Chem. Res. Toxicol*. 26: 767-774. doi: 10.1021/tx4000655

Enoch SJ, Madden JC, Cronin MTD (2008) Identification of mechanisms of toxic action for skin sensitisation using a SMARTS pattern based approach. *SAR QSAR Environ. Res*. 19: 555-578. doi: 10.1080/10629360802348985

Escher SE, Aguayo-Orozco A, Benfenati E, Bitsch A, Braunbeck T, Brotzmann K, Bois F, van der Burg B, Castel J, Exner T, Gadaleta D, Gardner I, Goldmann D, Hatley O, Golbamaki N, Graepel R, Jennings P, Limonciel A, Long A, Maclennan R, Mombelli E, Norinder U, Jain S, Capinha LS, Taboureau OT, Tolosa L, Vrijenhoek NG, van Vugt-Lussenburg BMA, Walker P, van de Water B, Wehr M, White A, Zdrazil B, Fisher C (2022) Integrate mechanistic evidence from new approach methodologies (NAMs) into a read-across assessment to characterise trends in shared mode of action. *Toxicol. in Vitro*. 79: 105269. doi: 10.1016/j.tiv.2021.105269

Escher SE, Kamp H, Bennekou SH, Bitsch A, Fisher C, Graepel R, Hengstler JG, Herzler M, Knight D, Leist M, Norinder U, Ouédraogo G, Pastor M, Stuard S, White A, Zdrazil B, van de Water B, Kroese D (2019) Towards grouping concepts based on new approach methodologies in chemical hazard assessment: the read-across approach of the EU-ToxRisk project. *Arch. Toxicol*. 93: 3643-3667. doi: 10.1007/s00204-019-02591-7

Gilmour N, Kern PS, Alépée N, Boislève F, Bury D, Clouet E, Hirota M, Hoffmann S, Kühnl J, Lalko JF, Mewes K, Miyazawa M, Nishida H, Osmani A, Petersohn D, Sekine S, van Vliet E, Klaric M (2020) Development of a next generation risk assessment framework for the evaluation of skin sensitisation of cosmetic ingredients. *Regul. Toxicol. Pharmacol*. 116: 104721. doi: 10.1016/j.yrtph.2020.104721

Hagan B, Shah I, G Patlewicz, (2025) Can graph similarity metrics be helpful for analogue identification as part of a read-across approach? *Comp. Toxicol.* 34: 100353. doi: 10.1016/j.comtox.2025.100353

Hartung T, Rovida C (2025) Mechanistic read-across comes of age: a comparative appraisal of EFSA 2025 guidance, ECHA's RAAF, and good read-across practice. *Front. Toxicol*. 7: 1690491. doi: 10.3389/ftox.2025.1690491

Kalgutkar AS, Gardner I, Obach RS, Shaffer CL, Callegari E, Henne KR, Mutlib AE, Dalvie DK, Lee JS, Nakai Y, O'Donnell JP, Boer J, Harriman SP (2005). A comprehensive listing of bioactivation pathways of organic functional groups. *Curr. Drug. Metab*. 6: 161–225. doi: 10.2174/1389200054021799

Klimisch HJ, Andreae M, Tillmann U (1997) A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. *Regul. Toxicol. Pharmacol*. 25: 1-5. doi: 10.1006/rtph.1996.1076

Kovarich S, Ceriani L, Fuart Gatnik M, Bassan A, Pavan M (2019) Filling data gaps by read-across: A mini review on its application, developments and challenges. *Mol. Inform*. 38: e1800121. doi: 10.1002/minf.201800121

Laroche C, Aggarwal M, Bender H, Benndorf P, Birk B, Crozier J, Dal Negro G, De Gaetano F, Desaintes C, Gardner I, Hubesch B, Irizar A, John D, Kumar V, Lostia A, Manou I, Monshouwer M, Müller BP, Paini A, Reid K, Rowan T, Sachana M, Schutte K, Stirling C, Taalman R, van Aerts L, Weissenhorn R, Sauer UG (2018) Finding synergies for 3Rs - Toxicokinetics and read-across: Report from an EPAA Partners' Forum. *Regul. Toxicol. Pharmacol*. 99: 5-21. doi: 10.1016/j.yrtph.2018.08.006

Lester C, Byrd E, Shobair M, Yan G (2023) Quantifying analogue suitability for SAR-based read-across toxicological assessment. *Chem. Res. Toxicol*. 36: 230-242. doi: 10.1021/acs.chemrestox.2c00311

Madden JC, Enoch SJ, Paini A, Cronin MTD (2020) A review of *in silico* tools as alternatives to animal testing: Principles, resources and applications. *Altern Lab Anim (ATLA)* 48: 146-172. doi: 10.1177/0261192920965977

Maggiora G, Vogt M, Stumpfe D, Bajorath J (2014) Molecular similarity in medicinal chemistry. *J. Med. Chem*. 57: 3186-3204. doi: 10.1021/jm401411z

Mansouri K, Taylor K, Auerbach S, Ferguson S, Frawley R, Hsieh JH, Jahnke G, Kleinstreuer N, Mehta S, Moreira-Filho JT, Parham F, Rider C, Rooney AA, Wang A, Sutherland V (2024) Unlocking the potential of clustering and classification approaches: Navigating supervised and unsupervised chemical similarity. *Environ. Health Perspect*. 132: 85002. doi: 10.1289/EHP14001

Mellor CL, Marchese Robinson RL, Benigni R, Ebbrell D, Enoch SJ, Firman JW, Madden JC, Pawar G, Yang C, Cronin MTD (2019) Molecular fingerprint-derived similarity measures for toxicological read-across: Recommendations for optimal use. *Regul. Toxicol. Pharmacol*. 101: 121-134. doi: 10.1016/j.yrtph.2018.11.002

Moermond CT, Kase R, Korkaric M, Ågerstrand M (2016) CRED: Criteria for reporting and evaluating ecotoxicity data. *Environ. Toxicol. Chem*. 35: 1297-1309. doi: 10.1002/etc.3259

Molander L, Ågerstrand M, Beronius A, Hanberg A, Rudén C, (2015) Science in Risk Assessment and Policy (SciRAP): An online resource for evaluating and reporting *in vivo* (eco)toxicity studies. *Hum. Ecol. Risk Assess.* 21: 753-762. doi: 10.1080/10807039.2014.928104

Moustakas H, Date MS, Kumar M, Schultz TW, Liebler DC, Penning TM, Salvito DT, Api AM (2022) An end point-specific framework for read-across analog selection for human health effects. *Chem. Res. Toxicol*. 35: 2324-2334. doi: 10.1021/acs.chemrestox.2c00286

Muldoon J, Moustakas H, Schultz TW, Penning TM, Bryant-Friedrich A, Botelho DJ, Api A M (2025) Advancing chemical grouping: Development and application of signature-based structure-activity groups for non-animal safety assessments. *Comp. Toxicol*. 36: 100391. doi: 10.1016/j.comtox.2025.100391

OECD (2025), *Guidance on Grouping of Chemicals, Third Edition*, OECD Series on Testing and Assessment, No. 418, OECD Publishing, Paris, https://doi.org/10.1787/b254a158-en (accessed 31 December 2025).Patlewicz G, Charest N, Ross A, Bledsoe HC, Vidal J, Faramarzi S, Hagan B, Shah I (2025) Building a compendium of expert driven read-across cases to facilitate an analysis of the contribution that different similarity contexts play in read-across performance. *Comp. Toxicol*. 35: 100366. doi: 10.1016/j.comtox.2025.100366

Patlewicz G, Cronin MTD, Helman G, Lambert JC, Lizarraga LE, Shah I (2018) Navigating through the minefield of read-across frameworks: A commentary perspective. *Comp. Toxicol*. 6: 39-54. doi: 10.1016/j.comtox.2018.04.002

Patlewicz G, Karamertzanis P, Paul-Friedman K, Sannicola M, Shah I (2024) A systematic analysis of read-across within REACH registration dossiers. *Comp. Toxicol.* 30: 100304. doi: 10.1016/j.comtox.2024.100304

Pestana CB, Firman JW, Cronin MTD (2021) Incorporating lines of evidence from New Approach Methodologies (NAMs) to reduce uncertainties in a category based read-across: A case study for repeated dose toxicity. *Regul. Toxicol. Pharmacol*. 120: 104855. doi: 10.1016/j.yrtph.2020.104855

Pestana CB, Leme DM, Silva EZM, Kiessig S, Firman JW, Kneuer C, Marx-Stoelting P, Cronin MTD (2025) Challenges and opportunities of read-across for the tumor promotion effects of microcystins. *Regul. Toxicol. Pharmacol.* 163: 105938. doi: 10.1016/j.yrtph.2025.105938

Roe HM, Tsai HD, Ball N, Oware KD, Han G, Chiu WA, Rusyn I (2025a) What does "success" look like in compliance check decisions by the European Chemicals Agency? The curious cases of accepted read-across adaptations. *ALTEX* in press. doi: 10.14573/altex.2505191

Roe HM, Tsai HD, Ball N, Wright FA, Chiu WA, Rusyn I (2025b). A systematic analysis of read-across adaptations in testing proposal evaluations by the European Chemicals Agency. *ALTEX* 42: 22-38. doi: 10.14573/altex.2408292

Ross A, Gombar V, Sedykh A, Green AJ, Borrel A, Kidd B, Phillips J, Shah M, Phadke D, Mav D, Balik-Meisner M, Howard B, Shah R, Kleinstreuer NC, Casey WM (2025) OrbiTox: a visualization platform for NAMs and read-across exploration of multi-domain data. *Front. Pharmacol*. 16: 1710864. doi: 10.3389/fphar.2025.1710864

Rovida C, Barton-Maclaren T, Benfenati E, Caloni F, Chandrasekera PC, Chesné C, Cronin MTD, De Knecht J, Dietrich DR, Escher SE, Fitzpatrick S, Flannery B, Herzler M, Hougaard Bennekou S, Hubesch B, Kamp H, Kisitu J, Kleinstreuer N, Kovarich S, Leist M, Maertens A, Nugent K, Pallocca G, Pastor M, Patlewicz G, Pavan M, Presgrave O, Smirnova L, Schwarz M, Yamada T, Hartung T (2020) Internationalization of read-across as a validated new approach method (NAM) for regulatory toxicology. *ALTEX* 37: 579-606. doi: 10.14573/altex.1912181

Sahlin U, Filipsson M, Öberg T (2011) A risk assessment perspective of current practice in characterizing uncertainties in QSAR regression predictions. *Mol. Inform*. 30: 551-564. doi: 10.1002/minf.201000177

Sapounidou M, Norinder U, Andersson PL (2023) Predicting endocrine disruption using conformal prediction - A prioritization strategy to identify hazardous chemicals with confidence. *Chem. Res. Toxicol*. 36: 53-65. doi: 10.1021/acs.chemrestox.2c00267

Schmeisser S, Miccoli A, von Bergen M, Berggren E, Braeuning A, Busch W, Desaintes C, Gourmelon A, Grafström R, Harrill J, Hartung T, Herzler M, Kass GEN, Kleinstreuer N, Leist M, Luijten M, Marx-Stoelting P, Poetz O, van Ravenzwaay B, Roggeband R, Rogiers V, Roth A, Sanders P, Thomas RS, Marie Vinggaard A, Vinken M, van de Water B, Luch A, Tralau T (2023) New approach methodologies in human regulatory toxicology - Not if, but how and when! *Environ. Int*. 178:108082. doi: 10.1016/j.envint.2023.108082

Schmitt BG, Roberts J, Kavanagh L, Dawick J, Frijus-Plessen N, Houthoff E, Klapacz J, Schowanek D, Shi Q, Zhang M, Hodges G (2025) Learning from experience: A retrospective analysis of read-across strategies for surfactants under REACH. *Regul. Toxicol. Pharmacol*. 162: 105884. doi: 10.1016/j.yrtph.2025.105884

Schneider K, Schwarz M, Burkholder I, Kopp-Schneider A, Edler L, Kinsner-Ovaskainen A, Hartung T, Hoffmann S (2009) "ToxRTool", a new tool to assess the reliability of toxicological data. *Toxicol. Lett*. 189: 138-144. doi: 10.1016/j.toxlet.2009.05.013

Schultz TW, Amcoff P, Berggren E, Gautier F, Klaric M, Knight DJ, Mahony C, Schwarz M, White A, Cronin MTD (2015) A strategy for structuring and reporting a read-across prediction of toxicity. *Regul. Toxicol. Pharmacol*. 72: 586-601. doi: 10.1016/j.yrtph.2015.05.016

Schultz TW, Chapkanov A, Kutsarova S, Mekenyan OG (2022) Assessment of uncertainty and credibility of predictions by the OECD QSAR Toolbox automated read-across workflow for predicting acute oral toxicity, *Comp. Toxicol*. 22, 100219. doi: 10.1016/j.comtox.2022.100219

Schultz TW, Cronin MTD (2017) Lessons learned from read-across case studies for repeated-dose toxicity. *Regul. Toxicol. Pharmacol*. 88: 185-191. doi: 10.1016/j.yrtph.2017.06.011

Schultz TW, Richarz A-N, Cronin MTD (2019) Assessing uncertainty in read-across: Questions to evaluate toxicity predictions based on knowledge gained from case studies. *Comp. Toxicol*. 9: 1-11. doi: 10.1016/j.comtox.2018.10.003

US EPA (United States Environmental Protection Agency) (2024) TSCA New Chemicals Program (NCP) Chemical Categories. US EPA, Office of Pollution Prevention and Toxics, Washington DC, USA. Available at: https://www.epa.gov/system/files/documents/2024-02/ncp_chemical_categories.pdf (accessed 30 December 2025).

Vrijenhoek NG, Wehr MM, Kunnen SJ, Wijaya LS, Callegaro G, Moné MJ, Escher SE, Van de Water B (2022) Application of high-throughput transcriptomics for mechanism-based biological read-across of short-chain carboxylic acid analogues of valproic acid. *ALTEX* 39: 207–220. doi: 10.14573/altex.2107261

Wareing B, Urbisch D, Kolle SN, Honarvar N, Sauer UG, Mehling A, Landsiedel R (2017) Prediction of skin sensitization potency sub-categories using peptide reactivity data. *Toxicol. in Vitro* 45: 134-145. doi: 10.1016/j.tiv.2017.08.015

Westmoreland C, Bender HJ, Doe JE, Jacobs MN, Kass GEN, Madia F, Mahony C, Manou I, Maxwell G, Prieto P, Roggeband R, Sobanski T, Schütte K, Worth AP, Zvonar Z, Cronin MTD (2022) Use of New Approach Methodologies (NAMs) in regulatory decisions for chemical safety: Report from an EPAA Deep Dive Workshop. *Regul. Toxicol. Pharmacol*. 135: 105261. doi: 10.1016/j.yrtph.2022.105261

Willett P, Barnard JM, Downs GM (1998) Chemical similarity searching. *J. Chem. Inform. Comp. Sci.* 38: 983–996. doi: 10.1021/ci9800211

Wohlleben W, Mehling A, Landsiedel R (2023) Lessons learned from the grouping of chemicals to assess risks to human health. *Angew Chem. Int. Ed Engl*. 62 :e202210651. doi: 10.1002/anie.202210651

Wu S, Blackburn K, Amburgey J, Jaworska J, Federle T (2010) A framework for using structural, reactivity, metabolic and physicochemical similarity to evaluate the suitability of analogs for SAR-based toxicological assessments. *Regul. Toxicol. Pharmacol*. 56: 67-81. doi: 10.1016/j.yrtph.2009.09.006

Wu S, Daston G, Rose J, Blackburn K, Fisher J, Reis A, Selman B, Naciff J (2023a) Identifying chemicals based on receptor binding/bioactivation/mechanistic explanation associated with potential to elicit hepatotoxicity and to support structure activity relationship-based read-across. *Curr. Res. Toxicol*. 5: 100108. doi: 10.1016/j.crtox.2023.100108

Wu S, Ellison C, Naciff J, Karb M, Obringer C, Yan G, Shan Y, Smith A, Wang X, Daston GP (2023b) Structure-activity relationship read-across and transcriptomics for branched carboxylic acids. *Toxicol. Sci*. 191: 343-356. doi: 10.1093/toxsci/kfac139

Yordanova DG, Kuseva CD, Ivanova H, Schultz TW, Rocha V, Natsch A, Laue H, Mekenyan OG (2024) *In silico* predictions of sub-chronic effects: Read-across using metabolic relationships between parents and transformation products. *Comp. Toxicol.* 30: 100314, doi: 10.1016/j.comtox.2024.100314

Yordanova DG, Schultz TW, Kuseva CD, Mekenyan OG (2021) Assessing metabolic similarity for read-across predictions. *Comp. Toxicol.*18: 100160. doi: 10.1016/j.comtox.2021.100160

Zhou YH, Gallins PJ, Rusyn I, Wright FA (2025) An approach to uncover significant direct and mediated relationships in multi-dimensional new approach methods (NAMs) data: A case study of hazard evaluation of petroleum UVCBs. *Sci. Total Environ*. 985: 179724. doi: 10.1016/j.scitotenv.2025.179724

Highlights

- Uncertainty in read-across assessments is categorised into seven criteria
- Read-across uncertainty has been characterised and the relative impact identified
- Assessment of (overall) uncertainty based on chemical structure and properties
- Simple and transparent template for uncertainty in read-across
- Tolerable uncertainty of the accepted read-across identified

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Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Mark Cronin is a current member, and Prof Terry Schultz a former member, of the Expert Panel for Fragrance Safety (https://fragrancesafetypanel.org).