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SCIENTIFIC OPINION

Guidance on the use of read-across for chemical safety assessment in food and feed

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

Read-across is a method used in chemical risk assessment to predict the toxicological properties of a target substance by using data from structurally and mechanistically similar substances, known as source substances. EFSA's Scientific Committee has developed an approach for using read-across in food and feed risk assessment. This method provides a step-by-step guide to applying read-across as part of a weight-of-evidence evaluation for individual substances. It includes an explanation of the key aspects to consider at each step of the read-across workflow, i.e. problem formulation, target substance characterisation, source substance identification, source substance evaluation, data gap filling, uncertainty assessment, conclusion and reporting. It highlights the importance of clarity, impartiality and quality to derive transparent and reliable read-across conclusions. A particular emphasis is placed on the analysis of uncertainty and whether the overall uncertainty can be lowered to tolerable levels by using standardised approaches, and/or additional data from new approach methodologies (NAMs). The guidance outlines methods to integrate data from NAMs to support read-across in the relevant steps, improving the robustness of the assessment. The ultimate goal is to equip risk assessors and applicants with a comprehensive framework to carry out read-across assessments systematically and transparently, thereby supporting the safety evaluation of chemicals in the food and feed chain.

KEYWORDS

data gaps, Food and feed safety, new approach methodologies (NAMs), read-across, risk assessment, target and source substances, uncertainty

†Deceased.

CONTENTS

SUMMARY

European Food Safety Authority (EFSA) Scientific Committee was requested to evaluate the use of read-across as an approach to address data gaps in the risk assessment of chemicals in food and feed and to advise on how new approach methodologies (NAMs) could be utilised to strengthen read-across justifications (EFSA-Q-2020-00413). Published literature and existing frameworks or guidance on read-across approaches were analysed to help develop the guidance.

Read-across is a method based on the concept of structure/activity relationship for predicting the toxicological properties of one substance (the target substance) based on data available from structurally and mechanistically similar substances (the source substances). The document details a structured workflow to standardise and justify the read-across approach useful in the context of data-poor substances, aiming to minimise uncertainties and ensure regulatory alignment. Moreover, it includes ways of integrating data from NAMs to support the read-across in the relevant steps.

The guidance is broadly applicable for the use of read-across in chemical risk assessments and in particular is intended to support the application of the read-across approach in the context of food and feed safety. The guidance seeks to equip the users (risk assessors and applicants) with the necessary framework to carry out read-across assessments transparently and systematically. It highlights the need for comprehensive documentation, scientific justification and a critical evaluation of uncertainties to support the conclusions of read-across assessments in a regulatory context.

The document includes the following chapters:

Chapter 1: Introduction introduces the concept of read-across, highlighting its significance as an alternative to animal testing for addressing data gaps in chemical safety assessments. It lays out the terms of reference, identifies the target audience and explains the use and degree of obligation of this guidance.

Chapter 2: Existing frameworks and guidance reviews the existing frameworks and guidance from organisations such as the European Chemicals Agency (ECHA) and the Organisation for Economic Cooperation and Development (OECD), which have laid the groundwork for the methodologies proposed in this document.

Chapter 3: Read-across context and requirements at EFSA presents the context and requirements for read-across at EFSA and its utility in regulatory risk assessments for food and feed.

Chapter 4: Read-across workflow describes the read-across workflow and provides practical guidance for performing read-across. It includes steps such as problem formulation, target substance characterisation, source substance identification, source substance evaluation, data gap filling, uncertainty assessment, conclusion and reporting.

Chapter 5: Applicability domain and characterisation of boundaries discusses the aspects of the applicability domain and characterisation of the boundaries for read-across and gives examples.

Chapter 6: Conclusions draws conclusions on the main aspects included in the guidance.

The document includes appendices (A–D) with detailed information on read-across processes, available in vitro methods, an uncertainty assessment template, case study examples and a glossary of relevant terms and definitions.

1 | INTRODUCTION

1.1 | Background and Terms of Reference

Read-across is an approach used in chemical risk assessment for screening, classification, prioritisation and hazard assessment of substances based on toxicological data of similar chemicals. It is one of the most common alternatives to animal testing (Cronin, 2013; ECHA, 2017c; OECD, 2014a; Patlewicz et al., 2013), providing opportunities for predicting toxicological responses for data-poor chemicals. A clear need for developing a framework and guidance on read-across within the European Food Safety Authority (EFSA) was identified at the 90th Scientific Committee plenary meeting on 17th of September 2018.

Terms of reference

This guidance was developed within a self-task mandate of EFSA's Scientific Committee that identified these objectives:

1. to develop a framework and guidance on the use of read-across in risk assessment, and
2. to identify the applicability domain (in terms of toxicological endpoints and chemical space) for the use of read-across in food safety.

1.2 | Target audience, scope, use and degree of obligation

This guidance is addressed to all those involved in EFSA's chemical risk assessments. The primary target audience is applicants and risk assessors.

Although its focus is on food and feed risk assessment, this guidance is broadly applicable to read-across in other areas of chemical risk assessment.[1]

This guidance is specifically applicable to individual substances and does not extend to chemical mixtures as such. While the primary focus of read-across has traditionally been on single target substances in the context of human health, its applicability for environmental risk assessments, as well as for complex chemical mixtures – including those of unknown or variable composition, complex reaction products and biological materials (UVCBs), food enzymes, other biological preparations and nanomaterials – are not within its scope and remains to be explored. Consequently, the guidance is not intended for direct application to these complex mixtures and materials, but it can be applied to individual substances that are part of a mixture.

Currently, read-across and the application of this guidance may not be considered acceptable as a substitute for already specified data requirements under existing regulatory frameworks. Nevertheless, a case can be made to use read-across as an additional supporting line of evidence in some situations, e.g. to fill data gaps[2] not covered in the dossiers of regulated products or to fill data gaps with publications in the open literature for non-regulated chemicals.

Two situations are foreseen (Figure 1):

- For regulated chemicals, the applicants generate data to support the read-across case (see also Appendix A, Figure A.1), if necessary and risks assessors evaluate the case.
- For non-regulated chemicals, the risk assessors use existing data to determine the feasibility of read-across, as new data cannot be requested.

[1]It should be noted that, in the EU, there are legislative requirements to submit toxicity data in several areas (pesticide active substances, food and feed additives, etc.).
[2]See Appendix E, the definition of 'data gap' in the context of this guidance.

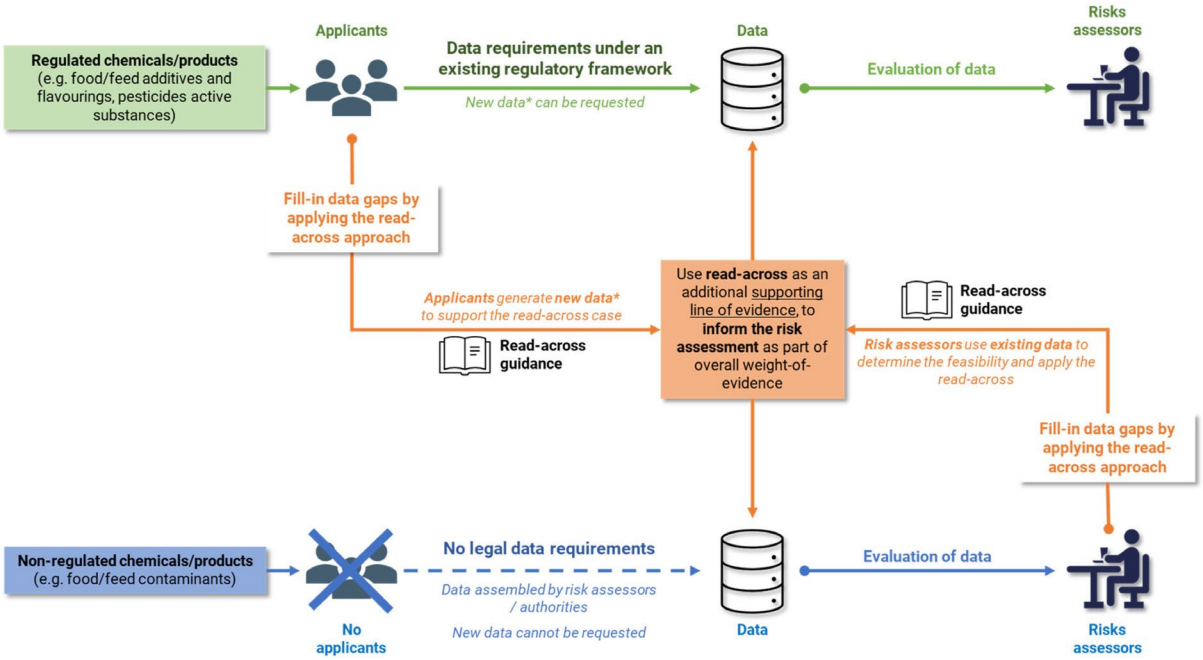

FIGURE 1 Situations foreseen in the application of the read-across guidance within EFSA's remit, including both regulated and non-regulated chemicals or products (the orange arrows and box represent the read-across option, when applicants or risk assessors may use this approach to support the overall risk assessment process). *New data refer to exploring NAMs first before considering any in vivo testing (explained in detail in Appendix A, Figure 1A).

Filling data gaps with a read-across approach can, in principle, be used for any endpoint, and the read-out contributes to the overall weight of evidence and reduces uncertainty in the risk assessment.

This document provides guidance on the general principles of the read-across approach and the use of NAMs in this context, but users have the flexibility to choose appropriate methods/tools and the degree of refinement in their application.

1.3 | Introduction to read-across

Read-across is a method used in chemical risk assessment to predict the toxicological properties of a data-poor target substance by using known information from one or more data-rich source substances that are structurally and mechanistically similar. It remains the most common alternative to animal testing to address data gaps (ECHA, 2023a; Rovida et al., 2020).

The technique is applied through two ways of chemical groupings, known as the analogue and category approaches (ECHA, 2017c) (see Appendix E):

(i) an analogue approach compares the properties of a substance with a limited number of closely related chemicals, namely, the target and source substances;
(ii) a category approach is based on the premise that structural similarity, which may include patterns or trends among several source substances, can predict the target substance's properties.

Read-across itself is the 'prediction' made within these chemical groupings – either a direct extrapolation of property information from source to target in the case of an analogue approach or one of several techniques (interpolation, extrapolation, etc.) in the case of a category approach.

There are several steps in the development of either approach. These steps are highlighted here and discussed in more detail in two key publications (Patlewicz et al., 2014, 2017). While there are variations, depending on which technical guidance or publication is considered, the general approach includes the following steps: problem formulation, data gap analysis, source substance/analogue identification and evaluation, data gap filling and uncertainty assessment. The final important step, to establish confidence in the application of the read-across approach, is to provide structured and adequate documentation.

In the context of both analogue and category approaches, the fundamental tenet of read-across is that substances which share similar chemical structures can be expected to elicit similar effects (as referred to in Appendix E). Hence, knowledge of one chemical (or a group of chemicals) can be used to predict the characteristics of similar chemicals. Since the intrinsic properties, propensity for metabolism, potential interactions and ultimate adverse effects of a chemical are encoded within its molecular structure, the knowledge and comparison of chemical structures for similarity is central to read-across. However, this should not be naively limited to a simple calculation of structural similarity indices, e.g. Tanimoto, Dice (Bero et al., 2017; OECD's Quantitative Structure-Activity Relationship (QSAR) Toolbox[3]) but also consider other aspects

[3]https://qsartoolbox.org/.

relating to physicochemical properties and chemical–biological interactions. Although structural similarity is the typical starting point in a read-across approach, a mechanistic understanding of chemical–biological interactions relating to the mode of action (MoA) also provides a basis for similarity in terms of mechanistic biological activity. Thus, structural and mechanistic profiles may offer, per se or in combination, a more solid basis for reliable read-across.

In brief, read-across allows the derivation of toxicity of a data-poor or untested chemical from the available experimental data on other structurally/mechanistically similar chemicals. Since read-across involves a number of steps, each of which may carry a certain level of uncertainty, it needs to be carried out in as transparent, standardised and unbiased a manner as possible to make the overall conclusions scientifically justified and reliable. Indeed, a properly carried out read-across can provide a useful means of addressing the gaps in, or absence of, toxicological information on a chemical to facilitate the assessment of its safety for use in a food/feed product. In this regard, although a number of frameworks have been proposed, the key challenge is still to establish a structured pathway that can be used to derive read-across conclusions with a tolerable level of uncertainty.

2 | EXISTING FRAMEWORKS AND GUIDANCE

The first substantive effort to describe the distinct and practical steps in formulating both analogue and category approaches stems from the development of the initial Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH[4]) technical guidance (ECHA, 2008) and the OECD guidance (OECD, 2007). Even with the subsequent revision of the OECD guidance (OECD, 2014a (under revision)), the main steps of the workflow have remained consistent. The only significant difference lies in the availability of new resources that can now be drawn upon when navigating the workflow:

- Computational software tools that can facilitate the construction of analogue and category approaches to make read-across predictions, e.g. the OECD QSAR Toolbox (OECD, 2014b).
- Structured databases containing traditional (e.g. in vivo) and NAM data streams that can be readily queried on the basis of chemical identifiers, e.g. eChemPortal[5], OECD QSAR Toolbox, EPA CompTox Chemicals Dashboard[6] (Williams et al., 2017).

Many of these resources are discussed in more detail by Pawar et al. (2019). Recent reviews of read-across tools have been discussed by Patlewicz et al. (2017) and Benfenati et al. (2019) (for details of the available tools, see Appendix A, A4.1).

The read-across approach described in the OECD guidance documents (OECD, 2007 and its revision OECD, 2014a), ECHA's guidance (ECHA, 2008) and ECHA's Read-Across Assessment Framework (ECHA, 2017c) (Table 1) have been adapted to other areas, e.g. several EFSA guidance documents include read-across approaches mainly applying the principles set out in the above-mentioned documents to specific regulatory contexts (e.g. food/feed area) (see Chapter 3). Moreover, these resources were used as the scientific basis for the development of the current guidance on the use of read-across in food and feed safety assessments.

All the guidance documents build on the framework and principles of the OECD (2007, 2014a) and therefore the approaches described are not in conflict. However, the more recent guidance document (OECD, 2014a) emphasises that, in addition to structural similarity, the utility of similar functional and biological activity and toxicokinetics, requirements for justifications and analysis of the uncertainties should also be considered.

TABLE 1 Documents providing guidance and describing read-across principles.

Document	Year	Context	Main topics	Target audience
OECD documents				
Guidance on Grouping of Chemicals, First Edition	2007	*Replaced by second edition*		
Guidance on Grouping of Chemicals, Second Edition (*under revision*)	2014	Hazard assessment of chemicals	Human health and environmental effects Mono-constituent substances Substances of unknown or variable composition, complex reaction products or biological materials (UVCBs)	Broad audience (industry, regulatory authorities, etc.)
Guidance on Grouping of Chemicals, Third Edition	2025	*Under development*		

[4]Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. OJ L 396, 30.12.2006, p. 1–849.

[5]https://www.echemportal.org/echemportal/.

[6]https://comptox.epa.gov/dashboard/.

TABLE 1 (Continued)

Document	Year	Context	Main topics	Target audience
ECHA documents				
Guidance on information requirements and chemical safety assessment. Chapter R.6: quantitative structure–activity relationships (QSARs) and grouping of chemicals	2008	REACH information requirements	Human health and environmental effects Mono-constituent substances Considerations for multi-constituent substances and UVCBs	EU Member State Competent Authorities REACH registrants ECHA expert assessors
Read-Across Assessment Framework (RAAF)	2017			

These are complementary resources used under different regulatory risk assessment frameworks. As such, one of the differences between the guidance documents relates to the regulatory framework and specific data requirements under which the approach is applied. For example, ECHA's guidance and framework are primarily aimed at supporting registrants to fulfil their obligations to submit the information required by REACH. ECHA's guidance (ECHA, 2008) Chapter R.6 provides general guidance on reporting (Q)SARs, grouping and applying read-across. The chapter is referenced in other ECHA guidance documents (non-exhaustive chronological list):

- Guidance on the Biocidal Products Regulation Volume III Human Health – Assessment & Evaluation (Parts B+C) (under revision) (ECHA, 2017b);
- Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a: Endpoint specific guidance (ECHA, 2017a; under revision);
- Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 (ECHA/EFSA, 2018);
- Guidance on information requirements and chemical safety assessment Appendix R.6-1 for nanoforms applicable to the guidance on QSARs and Grouping of Chemicals (ECHA, 2019);
- Guidance on the Biocidal Products Regulation Volume III: Human health Part A: Information requirements (ECHA, 2022b);
- Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7b: Endpoint specific guidance (ECHA, 2023a);
- Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures (ECHA, 2024).

2.1 | Utility of read-across in regulatory risk assessments

Since read-across can be used in different contexts, it is important to recognise the utility of the evidence derived from read-across in regulatory risk assessments of chemicals. In this regard, it needs to be noted that the current regulatory risk assessment schemes rely largely on data from 'officially validated' in vivo/in vitro methods, while other methods are subject to acceptance on a case-by-case basis following a rigorous appraisal. Despite the availability of high-quality databases, a wide range of in silico models and tools and guidance on best practice, the acceptance of read-across data by regulatory risk assessors is dependent on whether the read-across has been carried out according to appropriate guidance, and if sufficient detail, documentation and evidence have been provided to support the results. Assessment schemes, such as ECHA's RAAF, are also used to evaluate the robustness of read-across (ECHA, 2017c). The acceptability of read-across predictions may also vary according to the regulatory context, depending on the problem formulation.

For example, read-across can be used for the purpose of hazard-based classification and labelling within the CLP Regulation[7] as part of a weight of evidence (WoE) assessment using expert judgement.

The use of read-across in a regulatory context is also dependent on the availability of other data specific to the target substance and whether the read-across provides sufficient evidence for the relevant regulatory decision context. For example, the adequacy of the read-across for a risk assessment may differ from that needed in other contexts, such as for product development or hazard-ranking purposes. Another example is the case of food contaminants where other strands of evidence may not be available, making the use of read-across the only, or one of the few, available options.

Furthermore, in most cases, data generated by read-across need to demonstrate a robust justification to be accepted as a demonstration of the absence of hazard, although a weaker justification may be sufficient to confirm that the results indicate a hazard because the regulatory decision in such a case would still be on conservative grounds. However, the absence of a risk could be supported by clear evidence of absence of exposure and/or absence of biological interaction

[7]Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. OJ L 353, 31.12.2008, p. 1–1355.

leading to an adverse outcome. Both conditions should be carefully considered and taken into account when justifying a hypothesis for a negative read-across.

However, read-across results are generally more acceptable when they are presented as a supporting strand of evidence within an overall WoE analysis that has been assembled in conjunction with other lines of evidence, e.g. from in silico, in vitro and/or in vivo studies (EFSA Scientific Committee, 2017; SCCS, 2016, 2023a).

3 | EFSA'S REQUIREMENTS AND UTILITY FOR READ-ACROSS GUIDANCE

In the EU, the risk assessment of chemical substances in food and feed is covered by a range of regulatory frameworks spanning from data requirements for standard guideline studies to toxicity profiling of metabolites. EFSA's remit includes risk assessment and regulatory advice on the safety of a broad range of substances and foodstuffs, e.g. novel foods, food and feed additives, food contact materials and pesticides, as well as contaminants in food and feed. Thus, EFSA's remit covers several sectoral applications, encompassing a vast and chemically diverse range of substances that may require safety evaluation.

- For regulated food and feed product applications, there is generally a defined applicant who is responsible for providing the required toxicological data and exposure estimates for the risk assessment of a substance.
- In other instances, there is either no specific applicant or the substance has not undergone toxicological testing, or both. Examples include food and feed contaminants and botanical materials (that may comprise numerous substances), as well as metabolites and degradation products for which toxicological data are either partially available or absent. In the absence of a specific interested business operator, it is not possible to request that toxicological studies are carried out on such substances.

Even where testing a large number of substances would be possible, it could incur considerable economic cost, time and require the use of a large number of test animals. This is where alternative methods such as read-across can provide a basis for risk assessment.

Due to the breadth of uses and the requirements for toxicological information, combined with the paucity of data, read-across is seen as a possible way to fill gaps in the data. Read-across of toxicological data is already considered by EFSA in certain situations. For example:

- Risk assessment of smoke flavourings, e.g. application of read-across to assess the genotoxic potential of identified components in smoke flavouring primary products (EFSA FAF Panel, 2023a, 2023b, 2023c, 2023d, 2023e, 2023f, 2023g, 2023h). In these cases, the approach followed was recently described in guidance on the data required for the risk assessment of flavourings to be used in or on foods (EFSA FAF Panel, 2022).
- Risk assessment of feed additives, e.g. application of read-across to the assessment of the sesquiterpenes cis-thujopsene, α-cedrene, β-cedrene and the oxygenated derivative (+)-cedrol as major components of cedarwood Texas oil when used as a feed additive (EFSA CEF Panel, 2015; EFSA FEEDAP Panel, 2016, 2024).
- Risk assessment of pesticide active substances, for which data from in silico models and read-across could be considered sufficient to show that an impurity, a metabolite or a breakdown product is either toxicologically equivalent to the parent substance or lacks toxicological similarity to the parent substance, and therefore, additional experimental data might be needed. Thus, in a scenario of data-rich pesticide active substances, and where there are no legal requirements for specific toxicological data for metabolites, a read-across is generally considered before conducting additional animal studies for the metabolites (described in OECD (2009) (under revision)).
- Read-across has been used occasionally by EFSA to, e.g. predict the carcinogenic activity/potency (TD_{50}) of some contaminants, such as *N*-nitrosamines present in food (EFSA CONTAM Panel, 2023), application of read-across from perchlorate to chlorate for setting a tolerable daily intake based on human evidence using potency factors derived from a comparison of rat studies (EFSA CONTAM Panel, 2014, 2015).

To support such cases, there are guidance documents at EFSA that refer to the application of the read-across approach. However, these do not provide specific guidance on read-across, but mention read-across as an alternative and supporting line of evidence. A non-exhaustive chronological list includes:

- Guidance on the establishment of the residue definition for dietary risk assessment (EFSA PPR Panel, 2016);
- Genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019a);
- Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA Scientific Committee, 2019a);
- Scientific guidance for the preparation of applications on smoke flavouring primary products (EFSA FAF Panel, 2021);

- Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health (EFSA Scientific Committee, 2021);
- Scientific guidance on the data required for the risk assessment of flavourings to be used in or on foods (EFSA FAF Panel, 2022);
- Guidance document on the impact of water treatment processes on residues of pesticide active substances or their metabolites in water abstracted for the production of drinking water (ECHA/EFSA, 2023).

Considering the potential breadth of applications and diverse substances to which read-across may be applied, a generic, flexible and adaptable framework to implement this approach to filling data gaps is needed. However, for the use of read-across within a regulatory risk assessment framework, the key challenge is to develop a standardised workflow and accompanying guidance that together provide a scientific basis for the use of read-across in a rigorous, unbiased, reproducible and transparent manner. This means that the sources of data used are relevant and comprehensive, the selection of source substances (selected from the identified analogues) is based on a transparent structure–activity-driven principle/algorithm, and any expert judgement applied in this regard is justified on scientific grounds and is documented. The framework must be flexible enough to allow read-across based on multiple features (see Table 3), not just strict chemical space. Another key challenge for such a framework is to identify and define the applicability domain of a read-across – both in terms of toxicological endpoints and chemical space. Although read-across itself uses data from different sources (such as in silico, in vitro, in vivo), the outcome of read-across is based on interpolation/extrapolation of data from source substances. For use in safety assessments, the read-across data need to be integrated into a WoE analysis with other available data specific to the target substance (EFSA Scientific Committee, 2017). In order to implement the framework, this guidance aims to outline the different steps and provide appropriate background information and illustration to perform, justify and evaluate a read-across.

Finally, EFSA's scientific strategy 2027 (EFSA, 2021) calls for the development and integration of NAMs and omics for regulatory risk assessment. Recently, the EFSA roadmap, 'Development of a Roadmap for Action on New Approach methodologies in risk assessment' (Escher et al., 2022), identified the regulatory readiness of different approaches to filling data gaps, including read-across. It was concluded that read-across was indeed identified as an approach where the integration of NAMs has the highest level of readiness concretely related to EFSA's remit. Thus, a generic framework that integrates NAM approaches in read-across would be advantageous.

The present EFSA guidance, in addition to the general principles of read-across, lays out a stepwise approach, including ways of integrating different types of NAM data (in vitro assays predicting a similar metabolite profile, toxicodynamic, toxicokinetic endpoints and in silico data for physicochemical properties and any structural alerts) to support the read-across at relevant steps. It also provides guidance on performing a thorough analysis of the uncertainties pertaining to each step of the read-across, weighing the different lines of evidence and assessing the overall uncertainty.

4 | STEPWISE READ-ACROSS GUIDANCE

A structured workflow has been designed to support this guidance (Figure 2). It builds on the steps applied in the development of an analogue or category approach as briefly outlined in Section 1.3. It also considers the utilisation of NAM data and aims to clarify certain considerations regarding endpoint-specific data gaps. A summary of the workflow is provided in Table 2, with further elaboration in Sections 4.1–4.6. The individual processes are described in more detail in Appendix A.

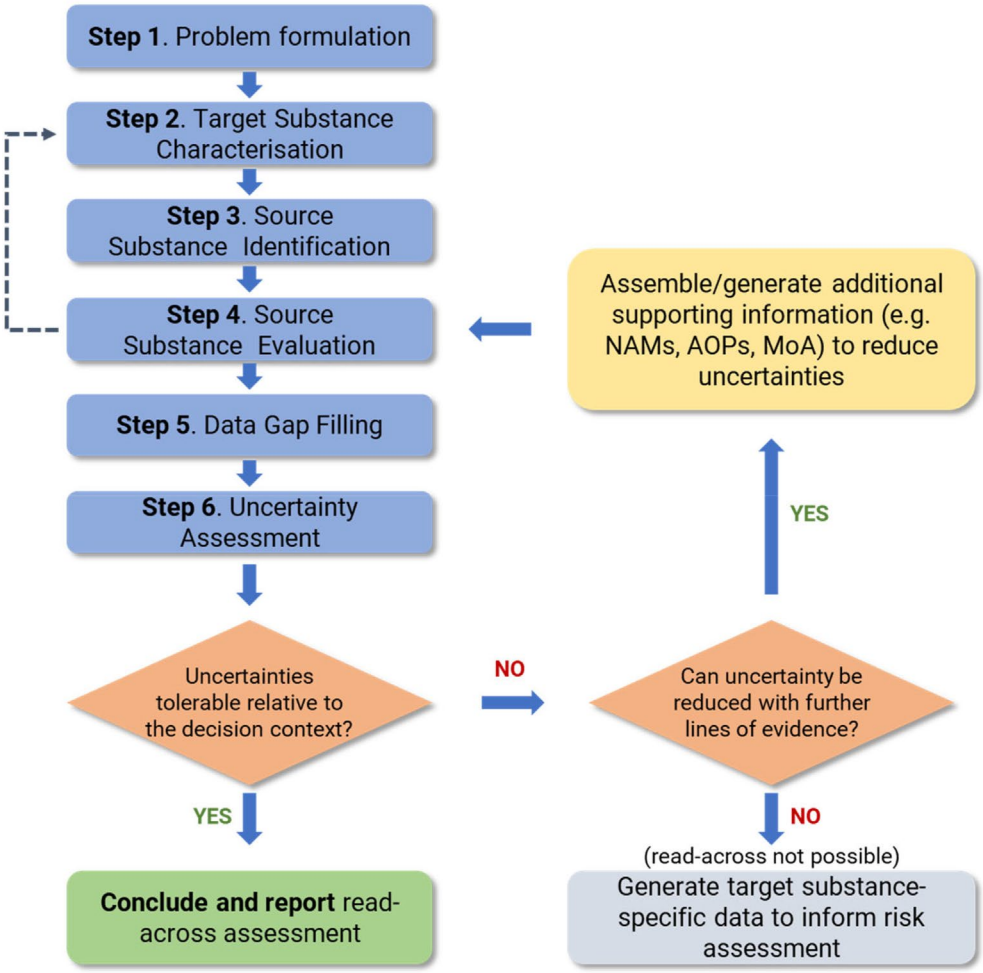

FIGURE 2 Overview of the stepwise read-across workflow. The problem formulation (step 1) defines the regulatory context of the read-across approach, including the level of tolerable uncertainty. The gathering and assessment of physicochemical, toxicokinetic and toxicodynamic properties of the target substance (step 2) leads to a read-across hypothesis and strategy. This hypothesis guides the identification of relevant source substances (step 3). In evaluating the toxicokinetic and dynamic properties of the source substances (step 4), dissimilarities might result in refinement of the source substances, adaptation of the inclusion criteria, inclusion/generation of additional data to support the read-across justification (yellow box) and potentially also a change to the read-across hypothesis. Thus, an iterative process may also occur at steps 4 and 2 (shown with a dashed line), ultimately resulting in a final hypothesis that the data gap filling (step 5) and the uncertainty assessment (step 6) are based on. Finally, depending on whether the uncertainties are acceptable or not, conclude and report on the read-across (green box) or fill the data gap with target substance-specific data (grey box).

TABLE 2 Summary description, data-related actions and outcome of the stepwise read-across workflow.

Step number	Step name	Aim and step description	Data-related actions	Outcome
1	**Problem formulation**	Defines what the assessment aims to address, and which options are available to achieve it. Identifies the data gap(s) for a specific endpoint for the target substance. Defines the objective of the data gap(s) to be filled. Describes the regulatory context and the level of tolerable uncertainty within the regulatory context.	Analyse and identify the data gap(s): the starting point is that the target substance has no or no reliable data for the endpoint of interest.	Data gaps are identified for a specific endpoint for the target substance. The regulatory context and the level of tolerable uncertainty within the regulatory context are defined.
2	**Target substance characterisation**	Considers the hazard information on the target substance and the data gap(s) that need to be addressed. Considers the hazard information and the data gap(s) that need to be addressed	Gather and organise all relevant information (in a data matrix) on the physicochemical, toxicokinetic and toxicodynamic aspects of the target substance, as well as in silico predictions.	Information organised in a data matrix. The evaluation of that matrix leads to a first read-across hypothesis that guides a read-across strategy and the selection of source substances to be performed in Step 3.
3	**Source substance identification**	Considers searching for candidate source substances that are similar to the target substance. Search strategy is based on structural similarity alone or also includes commonality in functional groups/reactivity/metabolic pathways.	Search for source substances according to search strategy.	A list of source substances for further evaluation in Step 4. Information organised in a data matrix.
4	**Source substance evaluation**	Evaluates the analogues identified in the previous step to better understand their (dis)similarity to the target substance and whether the differences might cause differences in adverse effects or potency.	Compile and document all existing in vivo, in vitro and in silico data. Identify signals/alerts that possibly indicate commonalities/differences. Evaluate the (dis)similarities. Available data should be evaluated for their relevance and reliability. Justify (de)selection of source substances scientifically. Assemble the information in the data matrix.	Data matrix is further populated. Conclusion on the further read-across strategy. Decision to continue with Step 5 of the workflow or start a new iteration gathering/generating further information to confirm the read-across hypothesis (Step 2).
5	**Data gap filling**	Based on the evidence collected in the previous steps, it defines the data gap filling strategy to support the read-across for predicting the endpoint(s) of interest for the target substance.	Apply a data-driven or expert-driven process for the read-across prediction. Finalise the data matrix.	The outcome depends on the problem formulation, e.g. point of departure, target substance properties.
6	**Uncertainty assessment**	Analysis of the uncertainties of the elements of the read-across (previous steps).	A qualitative and/or semi-quantitative analysis	The uncertainty of the finally predicted value/property for the target substance. The outcome will determine whether the read-across strategy can be/needs to be further pursued by reducing uncertainty. If the uncertainty is not acceptable, according to the problem formulation, further data generation/assembling may be pursued and integrated (step 4).

(Continues)

TABLE 2 (Continued)

Step number	Step name	Aim and step description	Data-related actions	Outcome
none	**Conclusion and reporting**	The aim is to provide a structured and sufficient documentation for the stated purpose. Report the conclusion of the read-across, along with description of uncertainties. The whole read-across process must be clearly documented and reproducible (rationale for strategy, scientific justification, search criteria, sources queried etc.).		Several tools for the generation of reporting templates are available (Appendix A, A7). Particularly notable are the OECD QSAR Toolbox, OECD integrated approaches to testing and assessment (IATA), and International Uniform Chemical Information Database (IUCLID) 6 (OECD Harmonised Templates), which can accommodate different types of data and undergo continued update and fine-tuning. Method description if non-guideline data have been used. The validity and reliability of the method(s) should be justified, along with a description of the uncertainties.

Data matrix

For the supporting data and to facilitate its integration into the read-across process, it is recommended to organise the different lines of evidence in a data matrix (Table 3) from the beginning of the process. The data matrix should be structured in a tabular format; examples are given in Appendix A, A7 (read-across documentation). Information should be collected for both the target substance and the source substances (analogues). If multiple source substances are identified, these should be arranged in a suitable order (e.g. according to molecular weight or logP). The cells of the matrix should be populated with the available information (examples are given in Table 3) and indicate whether data are available or unavailable.

This is essential for most steps of the read-across process and should begin at Step 2.

Application of such a data matrix:

- will highlight where supporting information for the different lines of evidence is missing;
- will provide the basis for the evaluation of the analogues, for the data gap-filling step and the assessment of uncertainties;
- will make it possible to draw conclusions on the feasibility of reading across the missing data of the target substance from those of the identified source substances.

TABLE 3 Examples of the type of information (with short descriptions) that can be included in the data matrix for both target and source substances.

Type of information	Short description or examples
Structures, identifiers and composition of the substances	e.g. chemical name, International Union of Pure and Applied Chemistry (IUPAC) name, chemical formula, chemical structure, Chemical Abstracts Service (CAS) registry number, simplified molecular-input line-entry system (SMILES), International Chemical Identifier (InChI), purity, impurity profile
Physicochemical properties and molecular descriptors	e.g. the partition coefficient (logP), molecular weight, water solubility, the distribution coefficient (logD) and pKa, all of which model likely bioavailability, similarity scores (Tanimoto or Dice)
Structural alerts	e.g. for potential toxicological hazards due to the presence of certain moieties in the chemical structure – such as those identified through OECD Toolbox profilers or other in silico systems
Data/proposal on mode of action (MoA), adverse outcome pathway (AOP)	If available or being postulated for the purpose of the read-across
In vitro/in silico data relevant to the MoA/AOP	The data should be arranged in the matrix according to the key events (KEs) (from (molecular initiating event) MIE, KE1, KE2 …. AO). The KEs might be measured by different methods which should also be stratified in the data matrix.
In vivo data	Both the data directly addressing the specific data gap, and other relevant in vivo data for the endpoint of interest (e.g. if the data gap is a 90-day study then studies of a longer or shorter duration). The in vivo data should be summarised describing the effects/adverse outcomes, as well as the reference points. Stratifying the in vivo data in the different effect/adverse outcome evidence lines could be considered. This approach is already applied for the assessment for the identification of endocrine disruptors of pesticides and biocides (ECHA/EFSA, 2018). This helps to organise the available in vivo data along MoA/AOP-based evidence lines, further helping the analysis of the data.
Kinetic data	In vivo, in vitro and in silico data on the absorption, distribution, metabolism and excretion (ADME)
Internal exposure data	Physiologically based kinetic model predictions on bioavailability and systemic/tissue exposures in humans/models, predictions from quantitative in vitro to in vivo extrapolation (QIVIVE) to contextualise in vitro toxicodynamic concentrations to in vivo concentrations.

Among the types of data indicated above, those reported in the table will vary according to the specific read-across analysis to be performed.

As such, the data matrix constitutes a source of supporting evidence. To facilitate the analysis of the read-across hypothesis, the data could be presented to also capture, e.g. the reliability of the study data (e.g. Klimisch score, Criteria for Reporting and Evaluating ecotoxicity Data (CRED) (Kase et al., 2016)), potency values, similarity scoring, etc.

Since the appraisal of the evidence is critical (EFSA Scientific Committee, 2017), systematic techniques can be considered, i.e. systematic review techniques/critical appraisal tools, like the US National Toxicology Program's Office of Health Assessment and Translation (US NTP/OHAT, 2019) approach or the Science in Risk Assessment and Policy (SCiRAP) platform[8]; these two examples of tools have the advantage that all types of evidence can be appraised within the same matrix.

[8] https://scirap.org/.

4.1 | Step 1: Problem formulation

The starting point for the read-across workflow is to formulate the problem, which defines what the assessment aims to address, and which options are available to achieve this. This should allow the 'user' of read-across, i.e. those making the read-across argument and collating the data, to identify the purpose and expectations of the assessment. Such a statement should allow any assessor to understand the purpose of the read-across – within the context of its respective regulatory framework and the endpoints being considered – including how and why it has been developed, as well as being able to determine the level of uncertainty that can be allowed to deem the read-across acceptable. The problem formulation also identifies and defines the data gaps for a specific endpoint for the target substance.

The problem formulation defines the boundaries of the evaluation, related to the effect, the exposure, the substance(s) and the associated level of tolerable uncertainty. Most of these considerations are also applicable to the evaluation of a group of substances, and the approach described here can also be used to define the boundaries of a chemical group.

In any case, the risk assessor should set the tolerable level of uncertainty considering the context of the read-across in the overall WoE. The main considerations for a risk assessor in this context may include, e.g. whether other lines of evidence are available for the substance, whether additional data can be obtained, whether the endpoint is of pivotal importance for the hazard assessment (such as carcinogenic, mutagenic and reprotoxic), and the urgency of the assessment. The purpose here is to determine whether there is sufficient confidence that the read-across is justified for the decision context and whether the read-across predictions of hazard and potency are robust. To illustrate the context further, the level of uncertainty that could be tolerated for a risk assessment decision would normally be lower than that for a risk-based prioritisation. In this context, Problem formulation (Step 1) is tightly coupled to uncertainty assessment (Step 6) where the overall uncertainty is assessed. This is also pertinent when considering potential strategies to reduce uncertainty or what other considerations (scientific, legislative, risk management) may facilitate or hinder that process.

In conclusion, the problem formulation step clearly defines what the assessment will address, and which options are available to achieve it. In that regard, it analyses the available data, identifies any data gaps for a specific endpoint for the target substance and defines the objective of filling those data gaps. The starting point is generally that the target substance has no, or no reliable, data for the endpoint of interest. It also describes the regulatory context and the level of tolerable uncertainty within the regulatory context.

More detail on 'problem formulation' is given in Appendix A, A1. The target substance is further characterised in Step 2.

4.2 | Step 2: Target substance characterisation

This step considers the hazard information and the data gaps that need to be addressed on the target substance. As such, once the target substance is unambiguously identified (CAS number, SMILES, InChI, etc.) and the aim of the assessment is specified in the problem formulation (Step 1) (e.g. endpoint of interest for the target substance formulated), all available information related to physicochemical, metabolic transformation, toxicokinetic and toxicodynamic aspects, in vivo, in vitro and in silico prediction for properties and any structural alerts for the target substance should be collected (see Table 3).

There are no specific requirements or limitations described in this guidance in relation to collection of data to characterise the target substance. It should reflect and address the needs identified in the problem formulation. However, it may be beneficial to consult multiple data sources and, where appropriate, gather evidence through a systematic review (EFSA, 2010). General principles for the adequacy of data should be followed, i.e. their relevance and reliability.

The information on the target substance, organised in a data matrix (see description above and Table 3), will clarify which information is missing in order to address the problem formulated and lead to a read-across hypothesis, and will guide the selection of an initial set of source substances. If the target substance undergoes biotransformation, the read-across hypothesis may also be based on the metabolite(s) if critical, and the characterisation will have to be repeated with the metabolite as the target substance(s). More detail on target substance characterisation is given in Appendix A, A2.

In conclusion, the evaluation of the obtained data matrix leads to the first read-across hypothesis, which guides a read-across strategy/selection of source substances performed in Step 3.

4.3 | Step 3: Source substance identification

Source substance identification is the process of searching for candidate source substances that are similar to the target substance. The data matrix can aid in collecting the information in a harmonised way (see description above and Table 3). Important here is the 'overarching similarity rationale', a term coined by Patlewicz et al. (2017) to capture the similar characteristics specified in the OECD guidance on grouping of chemicals (OECD, 2014a). It is important that this rationale, which defines the hypothesis at the basis of read-across, is stated and justified within the read-across, and this can be included in the problem formulation. The overarching similarity rationale dictates, to an extent, how this search will be conducted in practice. Thus, if there is a single adverse effect, this can be used to simplify the formulation of the rationale, focusing on that specific endpoint and any that are related. However, it is possible that for the source substances there are additional

mechanisms and biological/chemical processes, which may prevail over, or be faster than, the process that is in common with the target. Ideally, it may be safer to explore both a read-across approach which is focused on the endpoint of interest, and in parallel a general approach covering multiple perspectives. We can speak about supervised and unsupervised search methods, for these two cases. This strategy has analogies with what has been recommended by authorities for in silico models, applying methods which are both driven by specific mechanisms (such as expert-based models) or by machine learning or statistical approaches.

For a robust read-across, it is essential to combine multiple types of information. While structural and chemical similarity provides the starting point, additional relevant data must also be carefully considered, as outlined below. There are a number of ways to search and compare the target and the candidate source substances:

- Structural and chemical similarity: The chemical structure is at the basis of all the other phenomena. It can be measured in various ways, offering different numerical results, such as the Tanimoto index (see also Appendix A, A3). There is no unique approach and, in addition, the similarity may depend on the three-dimensional structure, which may be altered within the binding site, for instance. The structural similarity is always available, while the other similarity properties described below, in particular for experimental values, may not be available even though they can be predicted in many cases.
- Source substances based on physicochemical characteristics: Physicochemical characteristics may include identification of structural alerts, stability vs. chemical reactivity or conformation in space that might impact active-site binding, as they might be important features for the final effect. For instance, logP is indicative of a potential bioaccumulation, although it is not related to a particular toxic mechanism. Physicochemical characteristics may also affect toxicokinetic processes. The US Environmental Protection Agency (EPA) new chemicals categories, for example, has been codified as a profiler within the OECD QSAR Toolbox and permits similar chemicals to be identified based on structural features and in certain cases physicochemical rules based on their propensity to cause similar toxicities. Other examples are the software ToxRead and Virtual Extensive Read-Across (VERA) (see Appendix A, A4.1), which also shows specific physicochemical properties associated with the effect.
- Mechanistic analogues: The endpoint under consideration and the MoA (if known) can be used to identify analogues, and in many cases, this is at the basis of the supervised approach (see below). If a good hypothesis is available for the expected MoA, this is a key element. The OECD Toolbox has several mechanistic profilers that can be used to identify mechanistically relevant analogues. Other expert systems such as Derek Nexus[9], Toxtree[10], OCHEM[11] and VEGAHUB[12] provide structural alerts that can be helpful in searching for analogues based on similarity in likely toxicity. The Hazard Evaluation Support System (HESS) alerts and the P&G developmental and reproductive toxicity test (DART) profilers are other examples.[13]
- Common toxicokinetic profile: Different behaviour regarding absorption, distribution or excretion may result in differences between the target and the source substances.
- Metabolic analogues: Metabolic similarity remains difficult to assess, especially the quantitative aspects. In this regard, several cases can be expected, from dissimilar biological behaviour due to differences in generation of metabolites between target and source substance to when the target substance is a metabolite of a source substance, and thus, similar biological properties may be expected (ECHA, 2017c), as further detailed in Appendix A, A3.
  In the event that there is no information on the exact metabolite, but only the percentage of degradation, this information can still be used in a comparative way between the source and the target substance, to evaluate whether they are labile in the same way.
- Common breakdown products not associated with metabolism (e.g. environmental degradation): The stability/reactivity of the substance can play a major role. The rationale for an analogue/category approach might be to pair parent and breakdown products. This assumes that the toxicity data on the breakdown products would be expected to be representative of the toxicity expected from the parent substance itself following exposure. For this option, there are four possible cases, as further detailed in Appendix A, A3.
- Similarity of manufacturing process: An alternative similarity characteristic might be to group substances on the basis of manufacturing process. This is often the means by which UVCBs are grouped; an example being petroleum distillates based on boiling point ranges. Another example might be an impurity or intermediate product associated with the manufacture of a pesticide active substance. The different manufacturing processes may generate different impurities, with a role in the different properties, including adverse effects.

[9] https://optibrium.com/products/stardrop/modules/derek-nexus/.
[10] https://toxtree.sourceforge.net/.
[11] https://ochem.eu/.
[12] https://www.vegahub.eu/.
[13] It is to be noted that this guidance does not include all available tools, and listing a tool does not imply EFSA's endorsement.

4.3.1 | Supervised source substance identification methods

A search informed by structural features relevant to the endpoint(s) of concern would be categorised as a supervised approach. Most typically, the key element is the information on the MoA, so that similar substances with the same MoA are filtered. In this approach, a conceptual scheme is proposed, which defines the hierarchy of the steps that provoke the effect and it is compared to see whether the same scheme applies to the target and the source substances. Indeed, several characteristics of the substance may affect the final toxicological outcome, generating different behaviour for two substances that appear similar for the presence of the same structural alert, for instance. Any similar behaviour under this scheme provides an argument for similarity, while any difference indicates that a different behaviour between the target and the source substances is expected.

When the MoA of the target substance is known, the presence of the common MoA can be used to find source substances in conjunction with the structural similarity.

It is also possible that a substance manifests more than one MoA, identifying the critical lead effect which the read-across should address (see Step 4 and Appendix A, A5.1). In this case, the same procedure should be repeated for each MoA.

4.3.2 | Unsupervised source substance identification methods

If there is no information about the MoA that provokes the adverse effect, all of the metrics for similarity described above can still be used by gathering the data for the different options, including bioactivity similarity, e.g. omics. However, this will be associated with more uncertainty around the relevance of the specific features used for similarity.

The unsupervised approach may be used to predict non-specific toxicity (e.g. body weight changes) or no toxicity (above the limit dose, e.g. 1000 mg/kg body weight (bw)), but it often requires extensive evidence.

When adopting the unsupervised approach, it is preferable to use as many methods as possible, as listed at the beginning of Section 4.3.

4.3.3 | Gathering source substances with supervised and/or unsupervised approaches

The various ways to identify source substances are described above. It is preferable to initially apply a broad, comprehensive strategy, even following multiple hypotheses. Of course, this requires more effort, but the use of software tools can speed up the process. The information on a particular MoA associated with the target substance provides a strong basis for the search and simplifies the process. However, as observed above, it is possible that a substance has more than one MoA. It is also possible to imagine that not all MoAs are known. Thus, if possible, it can be useful to apply both the supervised and unsupervised approaches, to explore multiple possibilities.

In this way, a list of source substances is obtained. These source substances should preferably be characterised using multiple methods, as described above and this will make it possible to evaluate them from multiple perspectives. Indeed, the more commonalities that exist between the target and the source substance, the better. The way to evaluate the source substances is described in Step 4.

4.4 | Step 4: Source substance evaluation

This step of the read-across is a formal process to evaluate the source substances identified in Step 3 to gain a better understanding of their similarity to the target substance. It will also identify whether there are differences in structure, properties, toxicokinetics, metabolic transformation and toxicodynamics that could cause differences in adverse effects or potency. This is a pragmatic and expert-driven process reliant, in part, on the availability of data and information pertinent to the target and source substances, but equally on the availability of data to read-across.

The evaluation of source substances may be carried out manually by the expert or by using an in silico system (see also Appendix A, Table A.1). It is understood that expert evaluation may be required at any stage of the analogue evaluation. In either case, a stepwise process is needed to identify (and justify) those analogues that are most similar to the target substance in terms of:

- structural similarity, including assessment of molecular structure that influences toxicity;
- physicochemical properties;
- toxicokinetic properties;
- toxicodynamic behaviour;
- adverse outcome/endpoint-specific toxicological data.

The data matrix can aid in collecting the information in a harmonised way (see description above and Table 3).

Data and other information that are relevant, or related, to the endpoint being read across should be prioritised. For instance:

- More than one relevant toxicological effect has often been observed in in vivo studies of the source substances and in the characterisation; critical lead effect(s) should be identified and addressed systematically in separate lines of evidence (Appendix A, A5.1).
- If there is information on a known or plausible MoA, this will guide the identification of the important features to be used for similarity. In this case, in addition to structural similarity, where known and appropriate, the adverse outcome pathway (AOP) or the MoA can be identified.
- If the AOP or MoA is not known or available, advantage may be taken of the different metrics for similarity, but their role, relevance and contribution may be uncertain.

However, structural similarity only provides one component of the overall evaluation for use in a read-across. Other aspects relating to similarities in terms of the physicochemical properties, and/or the biological aspects are also important to be considered. Therefore, the evaluation of analogues potentially involves bringing together a variety of lines of information covering chemistry and biology.

A pragmatic starting point for the evaluation of source substances is to consider structural similarity. This is in line with the reasoning used by ECHA (2017c), who recommend the use of multiple perspectives for read-across. In order to formalise and quantify similarity between molecules, it might be necessary to use a metric of some sort. However, there is no single metric that can be ubiquitously applied across read-across scenarios. This is because different read-across cases may require consideration of different types of properties, effects and related information. Moreover, it is recognised that metrics are not comparable when they are calculated from different descriptor sets or molecular fingerprints, so care must be taken in their interpretation (Mellor et al., 2019). As one aspect of considering similarity, therefore, it may be appropriate to use one or more algorithms that can consider different means to evaluate overall chemical and biological similarity. However, even if there is no structural similarity between the analogues, they might be considered similar for read-across purposes if they share a common fate, metabolic pathway or toxicological MoA.

There are other elements that can have an influence on the adverse effects of analogues, and where expert evaluation is important. For example, it is well established that even a small difference in chemical moieties or the same moieties but at different positions in the source and target molecules, may lead to a change in toxicokinetic/toxicodynamic behaviour, and can potentially alter toxicological effects. This is sometimes called an 'activity cliff' (Pestana et al., 2022) and might make a read-across invalid. Although most of such 'subtle' changes in chemical structure could be captured by appropriate structural alerts, these examples highlight the crucial need for the expert to finally evaluate and decide on the most appropriate analogues identified by in silico systems.

Another aspect of expert evaluation is consideration of the reliability and relevance of the data associated with the selected source substances and whether the data were obtained by systematic techniques. It may be an advantage to apply tools/systems where all types of evidence (in vivo, in vitro, in silico) can be appraised within the same data matrix. Regardless of the different types of data, reporting and assembling the lines of evidence should be done in a consistent manner preferably applying a standardised ontology.[14]

Finally, in some instances, apart from assembling existing data, there may be a need to generate new supporting information from in silico methods. Moreover, in vitro methods can also be used here to support or confirm the suitability of selected source substances, further characterise potency trends across analogues, or to support the case for excluding certain source substances, e.g. when there are conflicting in vivo data.

In evaluating the toxicokinetic and toxicodynamic properties of the source substances, dissimilarities might result in the refinement of the source substances, adaptation of the inclusion criteria, inclusion/generation of additional data to support the read-across justification and potentially also changing the read-across hypothesis based on the assembled data. Thus, in practical terms, the process of identifying and evaluating candidate source substances might require several iterations, and refinement of the read-across hypothesis/strategy (Step 2).

In conclusion, once the relevant source substances have been identified (in Step 3), the assembled/generated data are organised in the data matrix. The final decision and reasoning to justify the source substance evaluation essentially requires expert opinion, because drawing a conclusion from automated systems based on different algorithms and/or structural alerts may not be sufficient (or possible) for a chemical read-across.

The outcome of Step 4 needs to be a decision on whether to continue with data gap filling in Step 5 or whether to refine the read-across with a new iteration to gather or generate further information to confirm the read-across hypothesis described at Step 2.

4.5 | Step 5: Data gap filling

Based on the evidence compiled in the data matrix, a strategy for filling data gaps is established to support read-across and enable prediction of the target substance's endpoint(s) of interest.

[14]Examples can be found at: https://bioportal.bioontology.org/.

Different strategies can be applied; either data-driven or expert-driven (preferable is a data-driven approach with the least contribution of expert judgement):

- Data-driven approach: There are various options for reaching a read-across prediction, such as:
  - the worst case, taking the lowest dose where the toxicity endpoint is observed amongst the source substances(s);
  - a value predicted from a statistically based trend analysis of the source substance values;
  - similarity weighted averages, as 'similarity' may be measured in different terms by different methods;
  - closest neighbour based on similarity; or
  - approaches to measure confidence in the predictions (e.g. Bayesian approaches).
- Expert-driven approaches could introduce additional uncertainty factors (EFSA Scientific Committee, 2018a, 2018b) or expert analysis of the available evidence, allowing a sound justification for selecting and scaling the most relevant candidate analogue.

Finalising the data matrix and deciding on the data gap filling strategy should enable a decision on:

- whether the data available on the analogues are sufficient to support a conclusion based on the read-across results, or
- whether at this point additional data need to be retrieved/generated before continuing.

The iterative character of the read-across process may, in certain cases, require a more accurate selection/analysis of analogues, with a corresponding change in the data gap filling strategy.

In the case of multiple reasons of concern, e.g. if there are multiple structural alerts, the evaluation should be repeated for each of them, possibly identifying similar substances containing the various reasons of concern simultaneously.

Additional information on approaches and tools used for data gap filling, including the use of NAMs data, is provided in Appendices 4 and 5.

4.6 | Step 6: Uncertainty assessment

The primary purpose of the uncertainty analysis in a read-across assessment is to determine whether the approach is scientifically justified and fit for purpose. This evaluation can be conducted either qualitatively, through narrative descriptions, or quantitatively, using probabilistic or semi-quantitative methods. The analysis helps to characterise the level of uncertainty at each step of the read-across process and assess whether it remains within tolerable limits, defined during problem formulation. Ultimately, this step confirms whether the read-across can be accepted, or if further data or refinement are needed to reduce uncertainty to a tolerable level.

The identification and characterisation of the uncertainty of a read-across serves at least three purposes:

1. to assess the overall quality and robustness of the read-across;
2. to identify uncertainties in a read-across that could be reduced by including further information;
3. to provide an indication of whether the read-across prediction is acceptable for a particular purpose.

Additional background information on how to characterise and address uncertainty in read-across is included in Appendix A, A6.

4.6.1 | Procedure for the assessment of uncertainty

The process for the assessment of uncertainty in a read-across is summarised in Figure 3. This assumes that a read-across has been completed, and that the read-across developer had the option to use data or information derived from standardised approaches (EFSA Scientific Committee, 2018a, 2018b) leading implicitly to a low, or acceptable, level of uncertainty for the given purpose. It also assumes that, if this was not the case, the read-across developer had attempted to reduce the level of uncertainty to an acceptable level (see Section 4.6.5 Reduction of uncertainty).

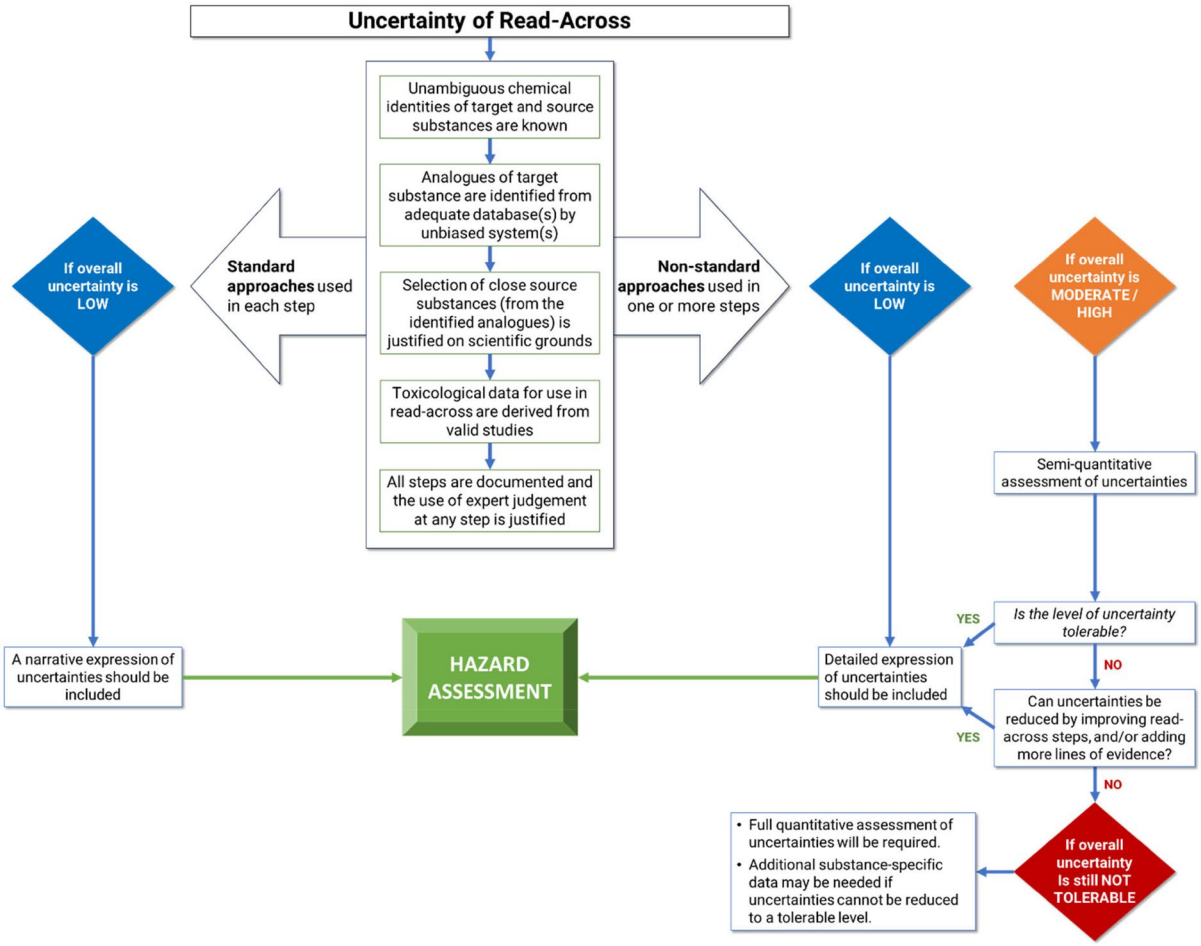

FIGURE 3 Summary of the processes of assessing the criteria for uncertainty of a read-across.

The procedure for the assessment of the uncertainty of read-across considers the uncertainties identified. It then allows the user to characterise the uncertainties in the read-across framework. (Semi-)quantification allows for the consideration of tolerable uncertainty, as well as an assessment with relevance to the standardised procedure. Generally, uncertainty is considered tolerable if it is within the definition established by the problem formulation and has a low impact on the read-across. However, higher uncertainty may also be acceptable in certain contexts of the read-across approach; e.g. when there is no, or only partial, information available and no applicant to provide new data.

It is expected that the uncertainties will be documented using an appropriate template (Appendix C) and that this will form part of the overall documentation of the read-across.

4.6.2 | Sources of uncertainties

There are implicit uncertainties at each step of the read-across workflow, and these should be characterised for each of these steps. The identified uncertainties require thorough characterisation to address all aspects of the read-across and ensure effective implementation within the framework. Briefly, the uncertainties identified can be related to, for example:

- identification and characterisation of the target and source substances;
- justification for the similarity between target and source substances;
- depth and appropriateness of the supporting metabolic transformation, toxicokinetic and toxicodynamic information;
- quality of the data to be read-across;
- documentation and reporting.

The main sources of uncertainty that are associated with the steps of the read-across are summarised in Table 4.

Uncertainties can be semi-quantified considering the definitions of uncertainty proposed by Pestana et al. (2021). These category definitions may be contextualised for a read-across to assign the level of uncertainty to one of the three (low, moderate or high) categories (these definitions are included in Appendix A, A6.3). A pragmatic way in this regard would be to first apply a simplistic check on the different steps and elements of a given read-across to see how the procedures had been followed, and what was quality of the data on the target/source analogue used in the read-across.

TABLE 4 Examples of typical sources of uncertainty in different steps of a read-across.

Steps in read-across workflow	Uncertainties with major impact on the read-across	Uncertainties with minor impact on the read-across
1. Problem formulation	– Insufficiently defined purpose of the read-across – Ambiguous description of the chemical structure/substance identity – Tolerable level of uncertainty not defined	
2. Target substance characterisation	– Endpoint-relevant existing data for target substance not identified. – The data gaps to be filled, i.e. a single endpoint or full toxicity profile, not stated. – Insufficient knowledge of the MoA	– An AOP (and the level of completeness) not available – A suitable MoA to support read-across
3. Source substance identification	– Definition of similarity rationale not provided – Absence of information on the quality of the databases searched	– Insufficient information on applicability of the databases searched – Detailed information on the criteria/algorithm used to find analogues not provided
4. Source substance evaluation	– Definition of structural similarities and dissimilarities between target and source structures not provided – Supporting physicochemical evidence not provided – Definition of toxicokinetic similarity not provided – Definition of toxicodynamic similarity not provided – Data quality not assessed or documented	
5. Data gap filling	– Insufficient integration of read-across with other sources of data to establish overall WoE	– Similarity in potency across the group/category not established – Supporting data not provided
Conclusion and reporting	– Documentation of read-across not provided, or only partially provided – Uncertainty assessment of read-across justification fails to address concordance between evidence and the read-across hypothesis	

4.6.3 | Characterisation and (semi-)quantification of uncertainties at each step

Uncertainty can be characterised at each step of the read-across process. It is recommended that the assessor characterises the uncertainties at each step as they progress through the assessment, as opposed to waiting until the end of the assessment.

4.6.3.1 | *Uncertainties related to Step 1: Problem formulation*

The analysis and acceptance of uncertainty in a read-across is important, yet not necessarily easy to address. The best way to address these issues is to capture the key questions at the outset during problem formulation, because the answers will largely depend on the context and the purpose of that specific read-across. Once an acceptable level of uncertainty is set, it can be used to assess the nature and the magnitude of uncertainty for each step of the read-across to decide whether the uncertainties of the read-across predictions are low enough to be acceptable for the given context and purpose.

4.6.3.2 | *Uncertainties related to Step 2: target substance evaluation*

In this step, the uncertainty analysis focuses attention on the requirements for the read-across based on the regulatory endpoints – these inherent aspects will factor into the overall uncertainty in terms of the sparseness of the data matrix.

4.6.3.3 | *Uncertainties related to Step 3: source substance identification*

Source substance identification will play a major role in the confidence of the read-across case. When evaluating potential sources of uncertainty for this step, consider the following aspects of the evaluation:

- What is the source inventory from which the analogues are queried?
- How expansive and representative is that inventory in terms of the chemistry of the candidate analogues that can be retrieved?
- How biased is the potential selection of the source inventory given the availability of good quality toxicity data to support the resulting read-across?
- Is the inventory pre-filtered based on certain types of toxicity data from specific sources?

Also consider the impact of the choice of structural representation used to conduct the structure searches and whether this can be systematically evaluated. For instance, many of the large databases of chemicals will be characterised by a specific chemical fingerprint approach to facilitate rapid searching. Uncertainty may arise if one fingerprint type is preferable to another. Furthermore, consider what impact the similarity metric will play in retrieving the most promising candidate source substance. The uncertainty assessment should discuss how many source substances were searched and whether there might be an optimal number or specific threshold for source substance identification. Using multiple similar substances with consistent results helps reduce the uncertainty; therefore, it is preferable to include more than one similar substance whenever possible.

4.6.3.4 | *Uncertainties related to Step 4: Source substance evaluation*

The demonstration of similarity is fundamental to the read-across process. It is often a complex procedure, drawing together various lines of evidence to be evaluated by an expert. A number of aspects of uncertainty relating to source substance evaluation have been identified (Schultz et al., 2019) and include:

- How are source substances evaluated based on their empirical and predicted data and what is the quality of the data informing that evaluation?
- How complete and authoritative are the data underpinning those source substances?
- How objectively can the different similarity rationales be assessed and what are the criteria for systematically determining the extent to which they are similar? For instance, differences in expected or predicted metabolic pathways – how different or similar should pathways be and how can this be quantified before the evaluation?

Another consideration is the extent to which each similarity rationale is evaluated independently and what collective impact these rationales have for the endpoint being read across. Is there an objective contribution that each similarity rationale plays and how does this differ depending on the toxicity endpoint and target substance under consideration? These factors also play a role in increasing the uncertainty of the overall read-across.

How are the read-across predictions ultimately made (e.g. worst case, interpolation or extrapolation)? What uncertainty is associated with a specific data gap-filling strategy and how can this be assessed to provide some bounds of uncertainty with the read-across prediction?

One last factor to consider is the analytical quality of the chemical characterisation of the target and source substances. How pure are the substances? What is the confidence that the samples of substances were adequately characterised? What impurities are there and to what extent might these mask the effects?

The similarity rationale considers the strength and validity of the hypothesis relating the target to the source analogues – what was the basis for the overarching rationale and what evidence (empirical or otherwise) supports that basis? Is the rationale underpinned by any mechanistic understanding for the endpoint being read-across? Is the rationale by virtue of a plausible metabolic transformation, a manufacturing process or is the rationale purely based on a structural similarity basis?

The similarity rationale also considers source substance validity – how relevant are the source substances relative to the target and the endpoint being read across? For this, considerations of the source substance similarity from both general and endpoint-specific contexts would contribute to the characterisation of any uncertainty. General considerations would include physicochemical, metabolic and reactivity similarity, consideration of the three-dimensional molecular configuration vis-a-vis a potential active-site binding – all factors that facilitate an assessment of whether differences in structure could contribute to a difference in biological activity/toxicity. A substance with a similar structure, but a different chain length, might result in a difference in its physicochemical profile that could impact its bioavailability potential. A substance with a similar reactive functional group but with different modulating side chains might nominally impart similar behaviours but the structural differences could culminate in a difference in potency. Branching patterns can impact likely transformation pathways or their kinetics. In isolation, none of these factors is necessarily directly linked to the endpoint of interest but termed 'general', simply because such differences can often be reasoned by virtue of reaction chemistry principles and have broader impact across many potential endpoints.

On the other hand, endpoint-specific considerations might consider factors that are pertinent to a particular MoA, e.g. oestrogen receptor binding where hydrophobicity and molecular volume might be significant chemical features or electrophilic reactivity for an endpoint such as skin sensitisation or genotoxicity where the molecular initiating events (MIEs) are coupled with reaction chemistry. Here, there is an understanding of the KEs within an AOP and assays characterising those upstream events can be closely associated with structural features.

Other similarity rationale-based considerations include the concordance of effects and potency per endpoint and across endpoints to the extent that this is possible to discern – can the trends observed across an endpoint and across a category be replicated for other endpoints? Is there consistency in the trends observed for the category members and across endpoints? An example here might be to consider the potency trend for a subacute toxicity endpoint and evaluate whether the same trend is mirrored across the subchronic endpoint. Another example might be to consider expected associations or consistencies between endpoints, e.g. is there convergence in the behaviour between eye irritation and skin irritation across category members or is there consistency in the potency and responses observed between Ames and skin sensitisation where there is an expectation that there is similarity in their reactivity underpinning the MIE? Data and similarity rationale are the overall considerations in evaluating the read-across justification.

4.6.3.5 | *Uncertainties related to Step 5: data gap filling*

The uncertainty relating to the overall completeness of the data matrix for the source and target substance should be considered. A sparse data matrix in terms of endpoints already filled or the target substance lacking most of the endpoints would contribute to greater uncertainty in the overall assessment relative to one where the pertinent data gap for the source/target substances was already filled. The rationale here being that there was sufficient connectivity between analogues and their underlying data to provide a strong foundation to support the read-across being proposed. A third factor to consider might be whether the approach relied upon an analogue or category approach. The analogue approach effectively extrapolates from a single source analogue to the target, whereas in the case of a category approach, interpolation and extrapolation across members of a category are feasible, the expectation being that a larger membership would permit trends to be investigated to support a read-across. Obviously, uncertainty is lower with interpolation as compared with the degree of extrapolation.

4.6.4 | Characterisation of overall uncertainty

The overall characterisation of uncertainty should be performed with reference to EFSA's Guidance on Uncertainty (EFSA Scientific Committee, 2018a). This considers all aspects of uncertainty and requires expert judgement to reach an overall evaluation. It is acknowledged that, where possible, uncertainty should be assessed quantitatively, using probability.

In the context of the read-across process:

- A quantitative assessment of uncertainty is encouraged but may not always be feasible;
- A narrative description of the overall uncertainty should be provided, especially when referring to the criteria in Appendix C (Table C.1);
- This narrative should address moderate and high levels of uncertainty in relation to tolerable levels for the read-across.

If all steps and criteria (Appendix C, Table C.1) indicate low uncertainty, the overall impact may be considered low. In such a case, a narrative account of any residual uncertainties should be sufficient to justify the validity of the read-across. Conversely, if appropriate procedures have not been followed at one or more steps, the overall uncertainty may range from moderate to high. A moderate level of uncertainty may arise from procedural shortcomings or a lack of confidence in the data. In these cases, a semi-quantitative assessment may be helpful to identify the main sources of uncertainty and determine whether these can be addressed.

For instance, uncertainty may be reduced by repeating a step using more standardised methods or by incorporating additional lines of evidence (e.g. NAM data). As an example, Schultz and Cronin (2017), in their evaluation of several read-across studies for chronic toxicities, identified key areas of high uncertainty, such as the quality of the data used, justification of similarity and toxicokinetics.

It is important to note that the uncertainty discussed in this guidance pertains to hazard assessment. In contrast, risk assessment may tolerate moderate or high levels of uncertainty, depending on other lines of evidence and/or the application of additional uncertainty factors.

4.6.5 | Reduction of uncertainty

When uncertainty is too high for a read-across to be considered fit for purpose, such as when non-standard procedures are used or data uncertainties are significant, the following steps should be taken:

- Further assessment and expression of uncertainty are required, which may involve: a semi-quantitative evaluation, or a quantitative statistical analysis to identify key sources of uncertainty;
- Inclusion of additional lines of evidence is essential, but it must be carefully evaluated whether this will effectively reduce the high uncertainty, and increase confidence to a level acceptable for regulatory purposes;
- If uncertainty remains high despite improvements: The read-across approach may not be feasible. Experimental testing of the target substance may be necessary.

The recommended read-across procedure is a stepwise process where the final outcome may strongly depend on adjustments based on feedback from the preceding steps, and – most importantly – from the consideration of the associated uncertainties (Figure 3).

Uncertainty may arise at each of the steps in the workflow where, upon identification, strategies to reduce those uncertainties need to be considered. Such strategies might take the form of applying assessment factors, performing further statistical analysis to better quantify the uncertainty and its impact in the overall decision or whether new data need to be generated. In the latter case, strategies to address uncertainty may well rely on the generation of other NAM data (see Appendix A, A5). For instance, collection of further information on the toxicity data, as well as NAM information to support the similarity hypothesis and toxicokinetics was found to reduce the uncertainty. Thus, further information would be

required, generally from NAMs in the first place, to reduce uncertainty, e.g. in conjunction with the available in vitro data (Escher et al., 2022) or in silico data (Pestana et al., 2021, 2022). If this is not sufficient to reduce uncertainties to an acceptable level, the risk assessor should ask the applicant to either revise the step(s) by following the standardised framework or generate target substance-specific data which inform the risk assessment.

Based on the specific regulatory context, different levels of uncertainty are tolerated. Typically, a qualitative outcome (e.g. from in vitro mutagenicity assessments) entails a description of the outcome and a type of uncertainty that will be different from that of a no observed adverse effect level (NOAEL) derivation, where quantitative parameters must be generated.

Closely linked to the above is the data gap analysis described in Step 2, which identifies the outcome to be predicted, and the characteristics required by the regulatory context. Assessment of the data gaps to be filled, and the specific regulatory need to be addressed, will facilitate the definition of 'acceptable' uncertainty. As with the assessment of the uncertainty, this will require an element of expert judgement to assist in the analysis of the consequences of an inaccurate prediction. For instance, lower uncertainty will be required in instances where an inaccurate prediction may result in an inadequate risk assessment.

Particularly sensitive to uncertainty generation is the source substance identification in Step 3. An assessment of similarity underpins the approach. The basic assumption is that the similarity in chemical structure, for instance, implies similarity in their biological or toxicological activities or properties. Uncertainty reduction first requires a careful scientific analysis of the specific case and consequent selection of the assessment of similarity criteria between target and potential source substance. The candidate analogues could then be refined by consideration of organic chemical functionalities. Such a strategy might be contrasted with a search that first compares core chemical scaffolds (as represented by SMARTS (SMILES arbitrary target specification)) and then using chemical similarity indices and/or functional groups to refine the search. A further action would be that of comparing experimental or predicted metabolic similarity.

One approach for reducing uncertainty in Step 3 could be through the identification of more analogues, e.g. by a different similarity index such as Tanimoto or Dice, or by a k-nearest neighbour (kNN) algorithm. However, although such an approach may find additional analogues, they may be relatively 'distant' in terms of similarity to the target substance and therefore not useful for a read-across. Instead, repeating the search in a comprehensive database (if not done in the first place) is likely to identify more relevant analogues that could have been missed out due to the limited chemical space covered in a smaller database. In any case, it needs to be remembered that even if a database search identifies only a few analogues, they might still be sufficient for the purpose of read-across as long as they are closely related to the target substance in terms of the threshold of the similarity index used.

Step 4 is the evaluation of the source substances identified in Step 3. To make a successful read-across prediction for an in vivo response, this step should take into consideration not only chemical structure similarity but also similarity in the hypothesised MoA and ADME profile. To this purpose, supporting information such as NAMs, AOP and MoA can be assembled/generated and then scrutinised. Multiple justifications of the read-across increase the confidence in the source substances found. Strategies to fine-tune the search for source substances and to reduce uncertainties could include targeted generation of new data based on sensitivity analysis. In the ideal case, this would entail the preliminary estimation of the benefit of acquiring additional information.

A case that deserves special attention is when read-across does not indicate a hazard. Such an outcome tends to be more meaningful if the target substance is part of a tested negative structural domain (i.e. populated by known and well-studied 'non-toxic' substances, supported by structural, physicochemical and/or functional parameters), as opposed to when the target substance is simply not a part of positive structural domain (in other words: similarity with proven 'non-toxicants' gives a robust indication of a lack of toxicity; lack of similarity with proven toxicants is no grounds to waive a concern for toxicity).

Filling data gaps in Step 5 is another critical step, where uncertainty can be controlled and reduced. The experimental data quality of source substance(s) should be carefully considered, with emphasis on expert judgement and by defining quality criteria (Appendix A, Figure A.1).

At the end of the read-across process, an iterative loop is included to assess whether the uncertainty exceeds what is needed for the overall decision context. Based on this final evaluation, uncertainty sources in the various critical steps can be re-analysed and reduced.

4.6.6 | Application of a standardised procedure

In the context of regulatory risk assessment, an example of low uncertainty in a read-across would be where each step had been carried out following appropriate procedures and any data used had been derived from 'standardised' methods (e.g. testing guidelines). The rationale for considering the level of uncertainty as low for such cases would be that the uncertainty relating to the procedural aspects and the data had already been addressed within the standardised protocol. This is in line with what is already practised by regulatory risk assessors, who may rely on data that have been derived from standardised methods, where available, e.g. from toxicological studies that have followed specific testing guidelines and good laboratory practice (GLP). Often, but not always, depending on the endpoint, uncertainty will be higher where data on hazard and potency were derived from 'exploratory', non-guideline studies, non-GLP or NAMs. Uncertainty of NAM data as such would rely on whether the method description is of sufficient clarity and detail to allow interpretation and use

of the data; for guidance, see NAM-related procedures, e.g on non-guideline in vitro test methods, good in vitro method practices, validation of QSAR models, regulatory assessment of QSAR models (OECD, 2014c, 2017a, 2018, 2023b, 2023c). In this regard, a recent external scientific report (Haase et al., 2024) has proposed a qualification system for NAMs for use in the risk assessment of nanomaterials, which may also provide pointers for application of the principles to other chemical substances used in the food and feed sector.

Therefore, it is important to ascertain that the following:

1. Information on target and source substances: In the first instance, an unambiguous identification and information on the chemical composition of both target and source substances is crucial for a read-across. Uncertainties at this step may arise from the lack of information on the exact chemical identity/composition, possible existence of a substance in different forms (stereoisomers, acid/salt, bulk/nanomaterial) and purity/impurity profiles of the substances used in toxicological testing. Such uncertainties need to be resolved so that unambiguous chemical structures are used in read-across. The useful pointers in this regard may include, among others, CAS number, chemical (IUPAC, ISO) names, EC number, SMILES, InChI and the information on key physicochemical parameters (in particular water solubility; logP, etc.).
2. Source substance identification: This step involves identification of those analogues or analogous categories that share structural and/or functional groups with the target substance. The main sources of uncertainty in this step relate to the use of a limited or selective search of databases, and/or the lack of use of a transparent method for searching the analogues. For a valid read-across, it is important that all possible analogues of the target substance or analogous categories with test data are identified and documented at this step. This essentially requires searching databases that are well defined, comprehensive and contain high-quality data. The use of a profiler for category identification also needs to be justified.

   In the initial searches for source substances, the threshold for similarity is generally set on a rational basis for a high level of structural similarity to keep the number of unrelated substances low. However, if no, or very few, source substances are identified, this may need to be lowered, while ensuring nonetheless that structural dissimilarities are not so high as to make the analogues unsuitable for use in read-across. Where information is available on the target substance in relation to physicochemical properties and toxicokinetic behaviour, further searches may include the use of profilers to find source substances or categories on the basis of functional similarities. The source substances identified on the basis of structural and functional similarities may be combined to look for any other analogues that may be similar both in terms of structural and functional aspects. It is important that the source substance identification is carried out in an unbiased manner using a transparent system/profiler that is based on a defined algorithm (e.g. fragment/fingerprint analysis), or a valid scientific principle (similarities in MoA and/or AOPs). It is also important that expert selection of analogues is not involved in this step in order to prevent bias.
3. Source substance evaluation: This step involves evaluation of each of the source substances identified above to select those that are most relevant for use in the read-across and to exclude the others on the basis of crucial structural dissimilarities. The main sources of uncertainty here include how certain source substances are selected for inclusion or exclusion in the read-across. To keep the level of uncertainty low, the evaluation for selection/exclusion of source substances needs to be justified on scientific grounds, e.g. selection on the basis of indices of structural/functional similarity, or exclusion on the basis of crucial structural anomalies. Expert judgement plays a part in the selection of source substances. However, source substance evaluation carried out entirely or largely on the basis of expert judgement would increase the level of uncertainty. Therefore, the use of expert judgement needs to be justified and documented.
4. Data aspects: Data should be fully documented and described. There is a particular need to assess and justify the quality of the data that are read across; e.g. using scoring schemes such as Klimisch or CRED (Kase et al., 2016), where available. The data to be read across should be of the quality expected for the information requirement to be filled. Uncertainties will arise where data of insufficient quality are utilised, or where data quality is not stated or justified. For other data, e.g. physicochemical properties and NAM data, uncertainties will relate to the description of the methodology, where possible quality assessment and relevance are not provided (e.g. see above for additional details on the uncertainty of NAM data and related OECD guidance).
5. Read-across conclusions: The conclusion should be in the form of an unambiguous statement that summarises the finding, i.e. the possibility of filling a data gap or completing an information requirement. The uncertainties here would be associated with a justified statement. The conclusion should state the target, the source, the endpoint, the overall uncertainty and whether or not the outcome carries a level of uncertainty which is within the tolerable level set for the read-across purpose during the problem formulation step.
6. Read-across documentation: Full documentation of the read-across process should be provided, see Appendix A, A7. This should include a full description of substance identity, tables fully detailing any relevant data, including the data points to be read across and, as required, relevant physicochemical properties and proper documentation for the performance of non-guideline in vitro methods according to OECD guidance 211 (OECD, 2017a) or the Toxtemplate (Krebs et al., 2019). The similarity hypothesis should be stated and fully justified. The level of documentation should be sufficient for the read-across process and data retrievable to be reproducible. This could, for instance, include computational workflows that capture the development of the read-across. Uncertainties in the documentation would include lack of definitive substance identity, data, metadata and a full definition and justification of the read-across hypothesis.

With these aspects in mind, a risk assessor will need to look at all the steps to weigh up whether the read-across was based on scientifically sound and justified approaches or whether there is a considerable contribution of non-standard or unjustified elements (Table 5).

TABLE 5 Key elements for the consideration of standards for each element of read-across.

Aspect of read-across	Standardised procedure	Non-standardised procedure
Target and source substances	The available data provide unambiguous information on chemical identity, structural features, physicochemical form, purity/impurity profile	Uncertainties over the exact chemical identity, possible existence of substances in different forms (stereoisomers, acid/salt, bulk/nanomaterial) and purity/impurity
Databases searched	Well-defined, comprehensive and high-quality databases	Selective, small or undefined databases
Identification of source substances	Based on an appropriate and justified algorithm or a defined scientific principle, inclusion/exclusion of the analogues for the endpoint of interest is justified	Algorithm/principle not defined, analogues selected based on personal choice and are not justified
Source substance evaluation	Analogue evaluation based on indices of structural/functional similarity – in conjunction with justified expert judgement*	Source substance evaluation based largely or entirely on expert judgement
Criteria for scoring structural similarity	Similarity based on scientific basis, or an algorithm (e.g. fragment/fingerprint analysis) – with expert judgement*	Similarity based entirely on expert judgement
Criteria for scoring functional similarity	Based on scientific principles/reasoning – with expert judgement*	Based entirely on expert judgement
Read-across conclusions	Full documentation supporting the justified conclusions	Poorly or non-justified conclusions

*A degree of expert judgement is always required to resolve equivocal or contradicting results. In such a case, a detailed justification is essential to explain the reasoning for any decisions made.

As indicated in Figure 3, each procedural inadequacy and/or non-standard element within the read-across process will push the uncertainty to a higher level. Thus, while a minor uncertainty should be acceptable for a read-across emanating from a fully standardised process, more and more in-depth analysis of uncertainties might be required for some or all steps if the read-across has been performed in a non-standardised way.

4.6.7 | Documentation of uncertainty

A template for the documentation of uncertainty is provided in Appendix C.

5 | APPLICABILITY DOMAIN OF READ-ACROSS

The applicability domain of a method or approach defines the chemical, biological or functional space within which the generated measurements, estimates or predictions can be considered reliable. The term is generally associated with non-testing NAMs, in particular in silico (Q)SAR-based models (OECD, 2014b, 2014c, 2023b), for which boundaries of the chemical/biological space covered can be defined based on the data sets used to build or test the model. In the case of read-across, the process of the identification of the boundaries is not to generate a general model but to identify the similar substances to be used for the read-across. Unfortunately, in this case, it is not possible to take advantage of the approach of the QSAR model (where the training set is specified a priori and the link between the molecular descriptors and the property is evaluated within the process of model development). On the other hand, the exploration of the domain for a read-across case aimed at fulfilling a regulatory objective proceeds using the metrics for similarity adopted in the specific case.

In general, a precise demarcation of the applicability domain is possible and important for a category-based read-across (ECHA, 2013), as it emulates a pattern or a trend from (several) substances identified as members of the category. In principle, this makes it possible to apply the same read-across outcome to more than one target substance as long as they meet the requirements for membership of the category. In contrast, setting an applicability domain for an analogue-based read-across is difficult, because the outcome of such an approach is limited to the specific target substance. Thus, read-across will have to be repeated for another target substance unless there is a (very) close structural/functional similarity between the two (Pestana et al., 2022).

The definition of the boundaries for read-across is not simple, including the difficulty in defining threshold values for similarity. Another difficulty is that it is not known whether the similarity function is linked to a pattern or trend. Furthermore, multiple similarity components can be used to reinforce the read-across procedure and in most cases the relative relevance of each similarity component is not known, or it is not known whether they are independent. Similar substances are included or excluded using different parameters for similarity, either applied sequentially or in parallel, within the process of

read-across. For example, omics data can be used for read-across, in a process parallel to that originating from the structural similarity.

If the MoA or AOP of the target substance is known, this can be applied in the initial phase of the analogue selection, and this can serve as part of the boundary definition. Account may also be taken of the existing grouping of similar substances used for read-across (e.g. from regulatory programmes or the literature). The information related to the structure is in any case a key component of the boundary definition (ECHA, 2013), which may be further strengthened by similarity in MoA or AOP relating to the property or properties under investigation.

It is common that, after the initial identification of the set of similar substances, filters are applied to refine the read-across process. These filters characterise the read-across boundaries. Several conditions are used for the identification of a cluster of substances for read-across, and these should be explicit. If the conditions for the boundaries are related to the presence of a certain moiety, such as molecular group or structural alert, these conditions are used as conditions for membership.

In Chapter 4, criteria to identify similar substances have been discussed. Multiple criteria for similarity are often applied. Some of them can be easily used to filter substances, e.g. the MoA. Thus, all substances with a certain MoA can be included, while others can be excluded, and this membership approach can be used to identify similar substances with clear boundaries. It therefore also clarifies the allowable structural differences among the category membership. In this way, the membership of a category can be ascertained to see if they fit within the applicability domain of the category. When applying thresholds, all substances defined by a threshold can be used to identify the applicability domain. This can be complex when the properties or features are expressed as continuous values or a range. In this case, if threshold values are used, these should be declared. For instance, the similarity measured in a certain way must be equal to or greater than a certain quantitative minimum; or the read-across is applicable for analogue substances with well-defined structural features such as a chain length between specific numbers of carbons. The justification should be provided, and it may be based on well-founded theoretical considerations or practical availability of a certain number of substances with experimental values.

In the case of continuous values, it is possible that the trend between the property to be evaluated for the target substance and the parameter used for read-across is not linear, and this should be explored. For example, this may show that the substances are not actually similar, the trend in the parameter does not hold for prediction of the property of interest or that the trend may hold (within break-points of the trend), but only within a subset of the substances and a different applicability domain. Thus, the applicability domain, even when related to a single feature (membership or physicochemical parameter) should be evaluated because there may be additional features affecting the outcome.

It is preferable to use more than one source substance for read-across. Indeed, if there are several similar substances, more matching features will be used for read-across, which will provide a stronger basis for it, and the applicability domain will have larger, better-defined borders. However, if a read-across is done only using a single source substance, the source and target substances must be very similar, and their differences should not be disposed to any major deviation. The most similar substances are those matching more features in common with the target substance. A more elaborate approach may assign a different weight to the membership depending on the relevance of the feature used for the similarity, for instance the same MoA. However, it may be difficult to assign these weights. Furthermore, when similarity is related to continuous values, the distance between the source and the target may be used to assign weights.

The applicability domain is more clearly defined when read-across is based on the interpolation of data. An example may be the case of source substances having longer and shorter aliphatic chains, compared with the number of carbons present in the target substance. Another example is when the source substances have logP values higher and lower than those of the target substance and logP can be shown to be relevant to the property of interest.

In principle, when read-across is done using multiple features, it should be possible for each of them to verify that interpolation is applicable, or at least that the same membership of the category is applicable. In practice, this may be quite difficult to obtain. Thus, different subsets of similar substances may be used for each feature, to double-check that the possible change to the value for a certain feature has not had a major impact in the outcome. In this way, the applicability domain is explored by considering different subsets of the applicability domain. It is also possible that two separate parameters used for read-across play an opposite role, and this higher complexity increases the uncertainty in the assessment. Ideally, source substances with different values (or memberships) for the two parameters should be used to verify when a certain parameter prevails.

It is important to recognise that a read-across case will always be endpoint-specific. Similar substances may behave similarly for one endpoint while having different outcomes for another endpoint. As such, the applicability domain of a read-across is also intrinsically dependent on the density of the chemical/biological data available for a given toxicological endpoint. Broadly speaking, the applicability domain of a read-across can be relatively wide for some toxicological endpoints, for which regulatory requirements for testing have led to large databases over the past decades. Examples include the Ames mutagenicity test, and to some extent, in vitro and in vivo micronucleus and chromosomal aberration tests. This is also the case for some other endpoints (e.g. skin sensitisation), for which MoA and adverse outcome pathways have been worked out.

Compared with these, databases on more complex toxicological endpoints (e.g. reproductive/developmental toxicity, repeated-dose chronic toxicity, carcinogenicity) lack sufficient data density to support a broader coverage of the chemical/biological space to allow a wider applicability domain for the read-across.

Since the key purpose of read-across is to provide data/information on substances for which experimental data on toxicological aspects are not available, it is meant to be complementary and inform the risk assessment, e.g. provides

supporting evidence which must be seen as part of the overall WoE and not as a substitute for the whole risk assessment process. This requires multidisciplinary expertise. Therefore, while interpolation/extrapolation of data/information is carried out on toxicological hazard for a read-across, the potency values are generally taken into consideration during risk assessment. However, if data on relative potency factors are available for the source substances for one or more toxicological endpoints, they can provide an additional element to consider in defining the boundaries of a read-across or a category.

In the context of this guidance, the EFSA Scientific Committee notes that the applicability of read-across in terms of toxicological endpoints and chemical space has so far been explored for genotoxicity and for repeated-dose toxicity (liver and developmental toxicity as well as general unspecific systemic toxicity), in both cases using pesticide databases (Benigni et al., 2020; Irwan et al., 2024).

The read-across workflow proposed in the present guidance has indicated effectiveness, as shown in the examples included in Appendix D.

6 | CONCLUSIONS

The concept of read-across derives from the tenet that similar molecules tend to have similar properties. The read-across methodology involves identifying data-rich (source) substances that are closely 'similar' to a data-poor/deficient (target) substance in terms of its structural and/or mechanistic aspects, and using data from the source substances to estimate toxicity of the target substance.

Taking account of the existing frameworks, guidance documents and any relevant proposals in the scientific literature, this guidance outlines a structured framework for applying read-across to estimate the toxicological hazard of a chemical substance to inform the assessment of its safety in the food/feed chain. In this regard, it needs to be noted that a read-across alone is not a substitute for risk assessment but is a means that may provide a useful line of evidence for hazard assessment, which is an integral component of risk assessment. Also, like other structure–activity relationship-based methods, stand-alone evidence from read-across may not be considered sufficient in a regulatory risk assessment to conclude on the toxicity of a target substance – in particular the lack of toxicity against a given endpoint. However, such data are generally more acceptable when presented as part of a WoE analysis in conjunction with other lines of evidence (e.g. in vivo, in vitro, omics).

The guidance explains each of the key steps involved in a read-across: problem formulation, target substance characterisation, source substance identification, source substance evaluation, data gap filling, uncertainty assessment and developing conclusions and reporting. It highlights that, depending on how the read-across has been executed, there may be a certain degree of uncertainty associated with each step. Taken together, these uncertainties may lead to a low, moderate or high level of overall uncertainty, which, depending on the context of the risk assessment, may or may not be tolerable. Therefore, a tolerance level for uncertainty must be set by the risk assessor at the outset during the problem formulation step. The guidance also discusses whether and how the uncertainty can be kept within the tolerable level. In this regard, it outlines the important considerations for each step that can be adhered to – for example, the use of standard procedures that might keep the overall uncertainty to a low level.

Assessing the possibility for obtaining data from NAMs to inform read-across by reducing uncertainties and to provide more support to justify the read-across is a recommended approach.

It also needs noting that the acceptance of read-across for use in risk assessments is context-specific. While the level of uncertainty is generally preferred to be as low as possible, a moderate or even a high level of uncertainty may also be tolerable in some situations – e.g. where no other data exist for the target substance, and/or the candidate source substances (selected from the identified analogues) are not similar enough to be useful for read-across.

While acknowledging the need for expert judgement at certain steps, the guidance stresses the importance that the read-across is performed in a transparent, standardised and unbiased manner and that justification is provided for the overall conclusions on scientific grounds. It also provides a few example case studies relevant to the areas within EFSA's remit where read-across has been applied to assess the risk of a chemical substance in the food/feed chain. However, since the main focus of read-across so far has been on single target substances in the context of human health, the applicability of this guidance for environmental risk assessment, as well as for complex chemical mixtures (including UVCBs, food enzymes and other biological preparations) and nanomaterials remains to be explored.

Finally, the guidance has recommended the use of read-across as part of a WoE approach based on current state-of-the-art methods. This is particularly relevant in the context of data-poor substances, where traditional testing data (e.g. in vivo) may be limited or unavailable. The ongoing work into developing new concepts, such as bioactivity/AOP considerations under 'Next Generation Risk Assessment (NGRA)', may open up other means of carrying out read-across in the future.

ABBREVIATIONS

2D/3D	two-dimensional/three-dimensional
3Rs	replacement, reduction and refinement
AC_{50}	active concentration 50 (concentration at which 50% of maximum activity is observed)
ADME	absorption, distribution, metabolism, excretion
AOP	adverse outcome pathway
CAS	Chemical Abstracts Service

$CL_{int,hep}$	intrinsic hepatic clearance
CLP	classification, labelling and packaging
CYP	Cytochrome P450
EC number	European Community number
ESAC	EURL ECVAM Scientific Advisory Committee
EURL ECVAM	EU Reference Laboratory for alternatives to animal testing
f_a	fraction absorbed
f_u	fraction unbound
GLP	good laboratory practice
HPV	high production volume
InChi	International Chemical Identifier
IVIVE	in vitro to in vivo extrapolation
IUCLID	International Uniform Chemical Information Database
KE	key event
KNN	k-nearest neighbour algorithm
LD_{50}	lethal dose 50
logD	log of the distribution coefficient
logP	log of the partition coefficient
MoA	mode of action
MIE	molecular initiating event
NAM	new approach methodology
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
OHT	OECD Harmonised Templates
PBK	physiologically based kinetic
pKa	acid dissociation coefficient
POD	point of departure
QIVIVE	quantitative in vitro to in vivo extrapolation
QSAR	quantitative structure–activity relationship
QSPR	quantitative structure–property relationship
RAAF	Read-Across Assessment Framework
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SAR	structure–activity relationship
SCCS	Scientific Committee on Consumer Safety
SMARTS	SMILES arbitrary target specification
SMILES	simplified molecular-input line-entry system
TD_{50}	median toxic dose
TEF	toxicity equivalence factor
TTC	threshold of toxicological concern
UVCBs	unknown or variable composition, complex reaction products or of biological materials
V_{ss}	steady-state volume of distribution
WoE	weight of evidence

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APPENDIX A

Description of the processes related to the read-across workflow

A.1 | PROBLEM FORMULATION

A starting point for the read-across workflow is the process of problem formulation. This should allow the 'user' of read-across, i.e. those making the read-across argument and collating the data, to identify the purpose and expectations of the assessment. Such a statement would allow any assessor to understand the purpose of the read-across – within the context of its respective regulatory framework – including how and why it has been developed, as well as being able to determine the level of uncertainty that may be allowed in a read-across to be deemed acceptable. The read-across problem formulation is a systematic approach that identifies all factors critical to a risk assessment and should be related to the overall problem formulation/assessment and question/subquestion, as outlined in the EFSA guidance on protocol development (EFSA Scientific Committee, 2023):

1. Translating the mandate(s) in assessment questions and making them operational.
2. Breaking down each assessment question into subquestions.
3. Determining the relative priority of the assessment questions/subquestions.
4. Selecting the overall approach.
5. Documenting the methods applied for the problem formulation.

Thus, in the context of using read-across, the problem formulation should be considered primarily to determine the scope of effort that would be merited and what resources would be required. In a wider context, read-across could be considered also where the testing of a large number of substances would be constrained by the time available and where the use of a large number of test animals could be needed.

The problem formulation should include a clear aim of the read-across, potentially including some or all of the following:

- A statement of the endpoint(s) to be considered during read-across.
- Definition of the chemistry, specifically identification of the data-poor target substance, and the source substances or a category.
- A description of the sources of data to be used, and the method/tools used for the selection of source substances or a category.
- The intended purpose of the read-across and level of certainty required.

In brief, the purpose of read-across can be stated as being a strategy to fill a data gap for [*INSERT AN* ***ENDPOINT*** *RELEVANT TO EFSA*] for [*INSERT* ***SUBSTANCE***] for the purpose of [*INSERT A* ***PURPOSE*** *RELEVANT TO EFSA*] (e.g. screening, grouping, hazard identification, hazard characterisation, risk assessment).

To achieve this, the appropriate level of uncertainty considered to be acceptable relative to the decision context should be specified (see **Chapter 4, Step 6**).

Further detail could be provided on the likely approach and mechanistic hypothesis, whether an analogue or category approach, known existing data etc., although in many cases, no existing data or information may be available.

A mandate received or data requirement defined by a specific regulation can also be considered here. These may contain information on the substance, regulatory question, legislation and time-frame available. The mandate or data requirement information should be used for the initial considerations in terms of problem formulation prior to initiating a read-across.

The following are considerations that may need to be addressed in the read-across problem formulation, noting that this is not an exhaustive list:

- It should be considered whether the sectoral legislation(s) referred to in the mandate allow the application of the read-across approach. Although this guidance aims at the widest possible application of the read-across approach in line with Directive 2010/63/EU (EU, 2010) and with the EU's Chemicals Strategy for Sustainability (CSS) commitment to move away from animal testing/reduce dependency on animal testing, the substance under consideration may fall under EU food/feed legislations that require a submission of substance-specific experimental data (see also **Chapter 3**). If so, the read-across approach alone is considered insufficient, although it can still be used to provide supporting evidence. An additional consideration to make is if the substance of interest is a metabolite or a contaminant/impurity of a main substance. Even if this substance falls outside the legal considerations applicable to the main substances, read-across approach may be applicable as supporting evidence. Lastly, the right of access to the necessary data to perform potential read-across (e.g. data protection and data ownership for the source substances) needs considering according to the applicable legislation.
- The second consideration is the identity of the target substance(s) and whether it is fully defined. An unambiguous substance identity of the target substance(s) is a prerequisite for read-across since the structural similarity is generally the starting point for the read-across.

- The scope of the mandate/assessment needs assessing also in terms of time and resource needs. It should be noted that read-across can be resource intensive (e.g. selection of appropriate source substances, possible need for additional supportive data generation and justification). In this context, it may be useful to assess whether alternative approaches are available to address the mandate/assessment. Depending on the scope of the mandate/data requirement, alternative approaches such as structure-activity based in silico methods or the use of the threshold of toxicological concern (TTC); (EFSA Scientific Committee, 2019b) approach may be more suitable, and this decision should be documented appropriately.

The above considerations will inform on whether a read-across approach could be applied. The subsequent considerations below may also need to be taken into account:

- Does the read-across address hazard identification or hazard characterisation to support a risk assessment?
- Is the read-across intended to support prioritisation for testing (e.g. if there is an existing concern)?
- Is there a comparison of the hazard profile or potency of two or more substances (e.g. parent substance vs. metabolite(s))?
- Is grouping considered (e.g. to support the grouping of chemicals for human risk assessment of combined exposure to multiple chemicals)?

A.2 | TARGET SUBSTANCE CHARACTERISATION: IDENTIFICATION AND SOLUTIONS

Analysis of missing information to cover the endpoint of interest identified in problem formulation consists of a wide-ranging assembly and evaluation of available information on the target substance, including structural characteristics, physicochemical and toxicokinetics/toxicodynamics data (in vitro/in vivo, etc.) related to the endpoint(s) to be predicted. This analysis will indicate whether information relating to some of the endpoints is missing for the target substance that can be addressed in the first place by testing (in vitro) or non-testing (in silico) NAMs or continuing to source substance evaluation.

Since a fundamental aspect of read-across is structural similarity, chemical composition and structural information should be well defined. An important element of a strong similarity justification is the definition and characterisation of all relevant physicochemical properties. This goal is achieved with the aid of available experimental information or reliable in silico predictions. The full set depends on the specific substance and the endpoint under investigation, and may include, e.g. melting and boiling point, water solubility, octanol/water partitioning coefficient, logP (LogP), volatility, e.g. as vapour pressure or Henry's Law Constant, particle size for powders, stability (e.g. in air, aqueous solution, pH, light) (Rovida et al., 2020).

Physicochemical and absorption, distribution, metabolism and excretion (ADME) data can indicate the bioavailability of the target substance or alert for possible bioaccumulation in the human organism. Similarly, (Q)SAR models can be used to estimate certain physicochemical parameters, supposed to play a role, in case that experimental data are missing. Furthermore, (Q)SAR models can also provide alert(s) for critical properties, such as chemical reactivity (e.g. binding with proteins or DNA indicating the potential for skin sensitisation or genotoxicity, respectively) or bioaccumulation. If available, the data matrix will also comprise data for 'related' in vivo endpoints.

Target substance characterisation therefore includes on the one hand evaluation of the completeness of data and nature of the available data to cover endpoint of interest and on the other hand it identifies metric(s) that could be important for the similarity analysis, e.g. if there is a clear MoA or an appropriate mechanistic profile (in terms of a common chemical class). The evidence can be retrieved from in silico, in vitro or in vivo mechanistic studies or be based upon common toxicologist knowledge.

A.3 | SOURCE SUBSTANCE IDENTIFICATION AND EVALUATION

A.3.1 | Strategies to identify source substances

Source substance identification is the process of searching for candidate source substances relative to a target substance. The target substance should have similar structural features and properties, as those of the source substance. Ideally, the pathway that leads to the adverse effect should be known. This knowledge should include the possible processes which may be part of this pathway, for instance adsorption, distribution, metabolism and degradation. Then, the main pathways and the possible deviations should be compared among the target and the source substances. The very first phase of this comparison, when applicable, starts from the production process. Indeed, in some cases, the production process can serve to identify similarities between the target and the source substance(s), for instance for the same preparation procedure, with the likelihood of generating the same impurities.

Some researchers have investigated the use of matched molecular pairs (Lester & Yan, 2021) as a means of searching for analogues that are likely to transform similarly and thus parent/metabolite pairs can be more readily identified. Others have derived focused structural alerts that codify metabolic transformations pertinent to a specific class of substances such that analogues returned are more relevant based on structural and metabolic considerations (Enoch et al., 2023, 2024; Enoch, Hasarova, Cronin, Bridgwood, et al., 2022; Enoch, Hasarova, Cronin, & Frericks, 2022). Others have used metabolic transformation similarity in conjunction with structural similarity to retrieve relevant analogues (Boyce et al., 2022;

Yordanova et al., 2021). Further research is still warranted to investigate the means of searching for analogues based on metabolic similarity.

The definition of chemical similarity depends on both the manner in which a substance is represented in terms of descriptors, as well as the similarity metric calculated (e.g. see **Appendix A**, **A4.1** more on kNN algorithm). In general, a chemical representation can be considered in terms of constitution, configuration and conformation. For source substance searching, the fastest and most efficient means of identifying relevant candidate substances relies on using a constitutional representation of substances. It must be recognised that a similarity score is dependent on the basis of the similarity, i.e. properties or molecular fingerprint (as well as the type of molecular fingerprint) (Mellor et al., 2019). As such, similarity scores are not comparable unless calculated from the same information using the same metric. This implies that any threshold in similar score set to identify or justify similarity should be set and interpreted independently of other thresholds.

Web-based tools such as ChemID, ChemSpider, CompTox Chemicals Dashboard use the Tanimoto index as the similarity metric, which is based on the substances' different chemical fingerprints. This explains in part why source substances retrieved are not necessarily the same and/or are associated with different similarity indices.

A key consideration for source substance searching relies not only on the metric and the manner in which the chemicals are represented but also the scope and size of the source inventory from which candidate source substances may be retrieved. A source inventory might be very large to encompass a diverse set of structures that are both real and virtual – or alternatively a source inventory could be constrained to only return source substances with associated toxicity data from specific sources or indeed tailored to only return source substances from curated and focused inventories of relevant chemistries, e.g. pesticide active substances but excluding drugs.

The underlying source substance inventory is the main contributing factor in why different source substances are returned from different web-based tools. Therefore, the quality and scope of the databases underpinning the different tools used to search for analogues is a major consideration.

While by far the most common source substance search is based on chemical structure using some form of chemical fingerprint, it is also possible to do a search for source substances on the basis of biological similarity, or both. Progress has been made in biological fingerprints using omics data, still in an exploratory stage (Kamp et al., 2024; van Ravenzwaay et al., 2016; Viant et al., 2024). In the simplest case, a target substance of interest with biological activity data would allow the search for similar substances with respect to their biological activity. A convenient means by which this can be done is to construct a fingerprint based on assay outcomes – either as a hit call or as a potency value, e.g. the ToxCast high-throughput screening (HTS) assays could be formulated in the form of a biological fingerprint whereby the assays would form the hits, the outcomes would indicate the presence or absence of that hit and the entire array would summarise the biological profile across a suite of assays. Using this as a probe and performing a pairwise comparison to an inventory of substances tested in the same suite of assays could then be used to retrieve the most similar substances on the basis of the assay profile.

As discussed below (see **Appendix A**, **A4.1**), tools that are used for the supervised approach can be also used if there is no specific clue for the MoA, still gathering different profiles according to different metrics. As an example, the GenRA tool as implemented within the CompToxChemicals Dashboard provides a means to search for similar analogues on the basis of ToxCast hit-call outcomes to return the top n analogues on the basis of ToxCast outcomes. The approach is extensible to consider not only hit-calls, but potency values represented as AC50s. The approach is generalisable to consider other types of high-throughput data such as high-throughput transcriptomics data (HTTr) or high-throughput phenotypic profiling (HTPP) (Tate et al., 2021). The search for source substances on the basis of biological similarity is not limited to HTS or similar, there is a whole related field of evaluating mechanistic similarity to elucidate MoA – see Lamb et al. (2006)) for the notion of connectivity mapping techniques that are complementary.

Searching for source substances is typically conducted in a stepwise manner whereby a candidate set of source substances are identified using structural characteristic and a series of filters are then applied to include or exclude substances based on their similarity in terms of reactivity, biological activity and metabolic characteristics in relation to the read-across rationale. This type of source substance identification strategy was termed as a filter approach (Helman et al., 2018), noting that the pool of source substances would be very focused on a specific structural scaffold, though not necessarily include source substances that might be relevant from a reaction or metabolic perspective. Alternatively, a search expansion approach is feasible whereby a search is performed to optimise the retrieval of source substances that are weighted based on several similarity characteristics at the same time. The weightings might be arbitrary, e.g. 50% structural 50% physicochemical or optimised based on a systematic analysis to elicit which weightings give rise to the best performance for the types of chemistries and endpoints of interest. Such a systematic analysis was undertaken to tune the weightings for physicochemical parameters in conjunction with Morgan fingerprints for different types of chemicals and in vivo toxicity study types (Helman et al., 2018).

The practical means of searching for source substances comprises several steps and is dependent on certain assumptions – whether a supervised or unsupervised approach is merited, as well as the quality and scope of the underlying source substance inventory and the characterisation of the substances being used. Similarity characteristics can be considered in a stepwise format as a series of filters or a search expansion approach where these characteristics are searched for at the same time. In either case, the source substance identification approach remains an iterative process depending on the substances retrieved and their subsequent evaluation as discussed in Step 4.

In the search for source substances, it is essential that the process of source substance identification is clear, transparent and reproducible. With regard to identification being reproducible, this can be achieved by either an algorithmic approach

or the application of sound scientific principle(s). An algorithmic approach would involve an automated process, for instance the entry of the target structure into a software tool that would identify source substances on the basis of computed similarity (which could include one or more of structural, property, metabolic or biological similarity) – the intention is that the operation in the software could be repeated independently and obtain the same results. Source substances could also be identified on the basis of prior knowledge or scientific principle. An example may be basing source substance identification on a known structural class, of e.g. pesticides, with a common 'core' or 'molecular scaffold' that is responsible for activity. Such a selection procedure must be stated as part of the problem formulation (Step 1) and be supported by evidence of a common MoA, and that the MoA is related directly to that molecular feature.

Importantly, it is essential to document the strategy undertaken so that the approach can be reproduced, including whether any source substances are deselected from consideration and the reasons why. For an algorithmic approach to source substance selection, details of the software (including version number) should be stated. In addition, the inputs into the software (such as the molecular identifier e.g. Simplified Molecular-Input Line-Entry System – SMILES – string or Chemical Abstracts Service – CAS – number) should be given, as well as the options selected e.g. similarity criteria or cut-off, type of similarity metric and calculation method. For source substance identification based on scientific principle(s), reporting should include the scientific principle(s) applied, and how they were implemented. For instance, if source substance were identified on the basis of a common molecular scaffold, this should be defined explicitly, with appropriate rationale. In addition, the documentation should include a description of the searching of databases with that molecular scaffold. The over-riding need for the detailed documentation is that the source substance identification process could be repeated independently.

The pathway leading to the adverse effect may involve a transformation of the original substance. The transformation may be a chemical one, such as hydrolysis occurring in water, or the transformation may be due to a biological process, such as metabolism. The same processes (for instance, metabolism) must occur for the target and the source substance. Ideally, the source substance should, for example, replicate the same series of events provoking the adverse effect.

In many cases, there are differences between the toxicological processes triggered by the target and the source substances, and these differences have to be addressed, to compare the properties of the source and target substance. For instance, if both the target and the source substance have the same AOP, this represents strong evidence of their similarity. However, attention should be given to metabolism and degradation processes, which may be different for the target and the source substance.

As anticipated in **Section 4.3**, in relation to metabolic similarity (metabolic analogues search option), below eight situations are outlined (see also ECHA, 2017c). However, in practice for each of the situations, there may be further scenarios and considerations. In particular quantitative aspects need to be considered. For example, quantitative differences in the amount of metabolite(s) formed relative to other metabolites as well as the parent substance, whether the metabolite(s) is driving biological activity and what would be the quantitative differences in this regard between the target and source substances:

1. The source substance but not the target substance generates a metabolite. This fact surely is a dissimilar behaviour. The overall final toxic effect of the two substances may be different if the metabolite is driving the toxicity.
2. The target substance but not the source generates a metabolite. The overall result is as in case 1.
3. The source and the target substances generate the same metabolite in quantitative aspects in relation to formation as such and relative to other metabolites formed, which indicates similarity.
4. The source and the target substance(s) generate a different metabolite, thus, for instance, the source substance metabolite might be considered as an analogue to the target substance. This indicates similarity, also related to the final effect, but not as strong as in the case 3. A modulation of the effect may be expected in this case due to the differences between the two metabolites.
5. The source and the target substances have different metabolic behaviour generating different metabolites. The two substances may be expected to be dissimilar and the source substance cannot be used for read-across, except in the case, where the metabolism of the parent substances is limited, and thus, this difference is not expected to drive the biological properties.
6. The target substance is a major metabolite of the source. In this case, if experimental data are available on the effects of the source, these are quite useful, and a similar behaviour can be expected (ECHA, 2008; EFSA PPR Panel, 2016). Examples for major metabolites can be a metabolite typically detected in urine of rats in ADME studies at levels ≥ 10% of the absorbed dose in both sexes (unless there are sex differences) or a metabolite in food of plant origin ≥ 10% of the total radioactive residue (TRR) and amount ≥ 0.01 mg/kg. The 10% value is not a rigid cut-off, and for the purpose of determining if a metabolite is major, it should be interpreted considering all available evidence.
7. The source substance is a major metabolite of the target. Also in this case, as in case 6, the experimental data on the source are useful. However, it is possible that the target substance shows additional effects; thus, the level of similarity is not as strong as in case 6.
8. Metabolic similarity may be defined with reference to the metabolic substructures of analogues. These have been shown to be definable using structural alerts derived from metabolic data within classes of pesticides such that activity data may be aggregated (Enoch et al., 2023, 2024; Enoch, Hasarova, Cronin, Bridgwood, et al., 2022; Enoch, Hasarova, Cronin, & Frericks, 2022).

On the common breakdown products not associated with metabolism, there are four possible cases:

1. The source substance generates a breakdown product, but not the target one. This indicates a dissimilar behaviour.
2. The target substance generates a breakdown product, but not the source one. This indicates a dissimilar behaviour.
3. The source substance and the target one generate different breakdown products through different pathways. This indicates a dissimilar behaviour.
4. The source and the target substances undergo the same degradation process; this represents an element for similarity, in particular if the breakdown products are the same.

In some cases, the information about the AOP or the metabolism may be missing. Thus, other features can be used to compare analogues, related to the physicochemical properties, or the chemical behaviour. The physicochemical properties can provide indications on the way of interaction between the chemical substance and the physical world, such as solubility, etc. The chemical behaviour, such as polarity, steric hindrance, flexibility, reactivity and other features, are associated with the physicochemical properties, but also the toxicokinetic and toxicodynamic properties, and thus, they represent further properties to be used for the comparison, in particular for cases of data poor substances.

Starting from the basic assumptions described above, there are many approaches for the identification of source substances. The problem formulation may establish which approaches are or are not appropriate for analogue identification.

The number of source substances may vary from a single substance to a large number of molecules. During the source substances identification procedure, it is common (although not required) that a large number of analogues may be identified. The number of source substances can be reduced through a selection process, sometimes referred to as subcategorisation. These analogues are identified and evaluated for being fit for purpose. Thus, the purpose here is to identify source substances and justify their similarity such that read-across may be acceptable.

Source substances identification procedures can be described as being supervised or unsupervised. These are defined as follows:

1. Supervised analogue identification procedure in this context refers to a custom search informed by some understanding or knowledge of the endpoint of interest and any structural features that are likely to be important in identifying analogues. This type of approach is more promising for endpoints where there is some mechanistic understanding. If the information on the MoA is well known, it will be more robust. A supervised search could also be customised by specific constraints in how a target substance was manufactured (substance identity, presence of impurities, etc.) or if there was prior hypothesis to tailor the search, e.g. metabolic rationale to link an ester target with an alcohol source substance since the latter would correspond to the transformation product.
2. An unsupervised analogue identification procedure makes no assumptions about pertinent features for similarity. This type of search would usually focus on using structure similarity as a means to identify source substances with an appropriate threshold. However, the most recent approaches take advantage of multiple similarity metrics, not only structural ones. Often, an arbitrary threshold from a similarity metric (such as a Tanimoto index) might be used as a pragmatic threshold to limit the number of analogues to a manageable size. This larger number of analogues would then be filtered on the basis of subsequent information.

In the unsupervised procedure, there are two main approaches to refine the analogues identified. The first is to search for analogues with structural similarity followed by filtering to narrow down and refine the substances (through a series of subcategorisations). The aim here is to ultimately return a set of the most promising candidates that are likely to share as many common aspects as possible of similarity. The filtering approaches are used to consider similarity in relevant structural alerts, experimental/predicted physicochemical properties, predicted metabolic profiles or even predicted toxicity endpoints. In this approach, the initial unsupervised process is coupled with further elements, which are based on the mechanistic elements; thus, this process is in practice hybrid. These subcategorisations aim to refine and distil the set of candidate source substances as far as possible before evaluating any experimental data for the candidate analogues. A key challenge of this approach is to cast a wide enough initial net to identify all possible candidate source substances. Another challenge is to avoid filtering out the candidate set to only a handful with limited experimental data or to those that are only structurally similar but may not share a similarity in other aspects – such as toxicity. At this point, another iteration of search and refinement might be merited.

The second approach that can be applied to the unsupervised identification procedure is known as a search expansion approach (Helman et al., 2019). In this case, the search aims to rely on several contexts simultaneously, e.g. a search where an equal weighting is applied on both physicochemical properties and structural characteristics. The challenge with this approach is twofold: (1) The assumption is that both contexts must be available for the starting inventory of chemicals being used to draw candidate analogues from and (2) inherent difficulties in determining how to select and adjust the weighting factors in a systematic manner. Regardless of the search strategy applied, the next step in the process is to evaluate the candidate analogues returned with respect to their experimental data.

In summary, the selection of the source substance identification method is dependent on a number of factors including the mechanistic knowledge of the read-across to be attempted (a mechanistic read-across may favour a supervised technique) and the structural basis to the read-across (finding structural analogues may be better performed with an unsupervised technique).

A.3.2 | Strategies to evaluate the source substances identified

Once source substances have been identified, it is necessary that their suitability for a read-across is evaluated. This is a crucial part of the justification of the read-across, as well as providing the evidence to establish the best source substance(s). Various methods can be applied to compare the target and source substances, but also in the case of groups of source substances under the category approach. Overall, this process should ensure that all relevant criteria for similarity are considered and that this process is demonstrated and adequately documented. The reasons for the inclusion or exclusion of source substances should also be documented and will serve as justification of the selection of particular analogue(s).

When comparing two substances, it is important to evaluate similarities, but it is also equally important to analyse dissimilarities, the role of the different components in the chemical structure, and whether they may increase or decrease the adverse effect(s). When definitive chemical structures are available, it will start with the understanding of the differences in chemical structure in terms of functional groups, position on the molecule of substituents, potential activating or deactivating groups, etc. Studies in this direction have been conducted, exploring the role of certain molecular moieties in the modulation of the effect, and these studies generated a new program for read-across (Viganò et al., 2022). This evaluation may be supplemented by consideration of relevant physicochemical properties. An initial analysis may be as simple as creating tables of properties, for instance as proposed by (Schultz et al., 2015).

Structural similarity may be based on certain chemical moiety/ies that have been associated with (adverse) biological activity. These structure–activity relationships (SARs) form structural rules, which can be captured computationally as structural alerts. Many chemical moieties can be those that are already known fragments and functional groups associated with a toxicological alert/AOP, for instance, to describe a general molecular property associated with toxicity such as protein binding (Enoch et al., 2011) or mechanistically based such as for hepatic cholestasis (Firman et al., 2021).

It is acknowledged, however, that creating comprehensive sets of alerts is time-consuming and requires an expert knowledge.

EFSA recognises that there are a number of considerations for the evaluation of source substances, which can be utilised as appropriate to the context. These considerations go across the information and properties of the target and source substances and can be summarised as relating to the quality (including concordance and consistency) of toxicity data to be read across, the overall assessment of similarity and dissimilarity between the candidate source substances and the target, as well as substance purity. More specifically:

1. Quality of toxicity data to be used in read-across: The underlying toxicity data associated with the source substance(s) should be evaluated in terms of acceptability for regulatory purpose. For instance, are the studies being carried forward in the assessment of adequate reliability and relevance, consistent in terms of guideline, exposure route and duration, etc.? This is important to ensure that a like-for-like comparison is made across the source substances and that any adjustments are made in exposure duration or that additional uncertainty factors are applied if the study quality is less than ideal. It is possible that, for some properties of source substances of interest, the experimental values are missing on some of the characteristics of the source substances used to support the read-across. In this case, in silico models may be used, for instance to obtain physicochemical properties, but also data regarding associated properties, such as molecular initiating events (Gadaleta et al., 2020) and toxicokinetics or metabolism.
2. Concordance and consistency of data for a group or category: Concordance and consistency can be evaluated across the source substances in terms of their potencies/responses, between the source substances and the target substance and across all relevant endpoints. In the first case, the toxicity potencies/outcomes across the source substances should be consistent, either in terms of being constant or in terms of following a trend, and this has to be checked. For example, a set of source substances that are consistent in terms of their irritation profile or the irritation potency changing in relation to a discriminating factor such as molecular weight. Consistency might also capture commonality in effects. Consistency in effect among source substances provides confidence in the WoE. Many of these similarity context considerations were captured by Schultz et al. (2015)).
3. The impact of the similarity context, if factored into the source substances identification step: Certain similarity considerations might be considered of higher importance relative to the endpoint of interest, e.g. physicochemical properties vs. structural similarity vs. metabolic similarity. This is usually part of the WoE assessment made by the assessor in formulating the read-across justification.
4. Substance purity: Substance purity to qualify the experimental data, i.e. are there significant impurities that could impact or confound the toxicity observed and would these likely to manifest themselves in the target substance?
5. Assessment of the dissimilarities between the candidate source substances and the target: Are any dissimilarities and differences likely to impact the toxicity observed significantly or modulate the potency? For instance, methacrylate and acrylates differ by the presence of a methyl group which reduces the reactivity observed, and therefore, their potency is reduced in some adverse outcomes, while the reaction mechanism remains common to both acrylates. If possible, the role of the different molecular moieties should be investigated, if the different moieties may cause different behaviour in the target substance compared to the similar substance. There are specific software programs that may assist to scrutinise the modulation role of the molecular moieties, such as ToxDelta, QSARpy and VERA (Ferrari et al., 2018; Golbamaki et al., 2017; Viganò et al., 2022).

Extensive technical guidance is available which could be applied (ECHA, 2008; OECD, 2014a), and which describes as part of the workflow of category/source substances development the assessment of source substance(s) similarity. A number of other key studies have illustrated how read-across source substances can be evaluated. The main relevant findings are summarised below:

- '*(Wu et al., 2010) presented the systematic expert-driven process to evaluate source substances for read-across in SAR-based toxicological assessments. In outlining the overall source substances identification and evaluation process, it describes the ranking of source substances in more detail with respect to different similarity contexts. The first step of the overall workflow relies upon a chemistry expert(s) to review the target substance in conjunction with other tools/resources (such as expert systems, structure searchable databases, literature, etc.) to devise the optimal search strategy that will take into account key functional groups and features, and how these might impact the physicochemical, reactivity or metabolic profiles. Based on the search performed, the results are then filtered to retrieve only those source substances with relevant toxicity data*' (Patlewicz et al., 2018).
- '*An assessment of the source substances is then performed to evaluate their suitability based on their structural, physicochemical characteristics, reactivity and metabolism. For the initial search, a Tanimoto threshold of 0.75 is used as a default to limit the number of structurally similar analogues retrieved (this threshold may be modified). Evaluating the structural differences between the source substances and the target is then performed to appreciate whether any of those differences would lead to a significant difference in the reactivity and toxicity anticipated. This evaluation is performed to evaluate: (1) the commonality of structural alerts (such as those contained within expert systems such as Derek Nexus, Lhasa Ltd); (2) the commonality of key functional groups (that would be critical for driving the reactivity and sites for metabolism); (3) the commonality in position of double bonds and, (4) the effects of additional functional groups. This is followed by an assessment of the similarity of the physicochemical characteristics of the source analogues relative to the target e.g. logP, molecular weight, water solubility, as well as logD and pKa, all of which model likely bioavailability. The last consideration is the similarity of the (known or predicted) metabolic profile between the target and source substance*' (Patlewicz et al., 2018).
- '*(Wang et al., 2012) described a tiered surrogate approach based on identifying three main types of potential surrogates (source substances) to ultimately select the best one for use in a quantitative risk assessment. The first step involves understanding what is known about the target substance in terms of its available data and any inferences that can be made about its reactivity, metabolism and toxicity. If no adequate repeated-dose toxicity information is available, a search is made to identify surrogates based on structural, metabolic and toxicity considerations. For a first type of surrogate, structural considerations include identifying structurally similar substances using the Tanimoto similarity index with a suitable cut-off. Similarity in key functional groups and reactivity making use of structural alerts is also considered. Metabolic surrogates are the second type and include metabolic precursors, metabolites and (bio)degradation products/precursors. Literature data or toxicokinetic testing will inform this surrogate type. The third type of surrogate is toxicity-like – based on, e.g. similar dose-response curves based on a toxicity equivalence factor (TEF) or relative potency factor (RPF). Additionally, similarity considerations with respect to common target organs, toxic effects, MoA and group membership (as in well-defined chemical classes/mixtures) are evaluated when identifying this surrogate type. The tiered surrogate approach is reliant on the surrogates identified being associated with established reference/toxicity values from regulatory agencies in order to compile a pool of data-rich surrogate candidates with good quality repeated-dose information. Physicochemical parameters are collected and added to the pool of information. A WoE approach is then used to rank the surrogates on the basis of structural, metabolic and toxicity similarity considerations. In this approach, emphasis is given to biological similarity (toxicity/toxicokinetic) over structural similarity*' (Patlewicz et al., 2018).
- Patlewicz et al. (2013) structured a workflow for category/analogue development around the category/analogue reporting format that is described in the OECD (OECD, 2007) and the ECHA guidance (ECHA, 2008). A series of steps of read-across development were articulated: decision context; identification of data gaps; the overarching rationale for analogues identification; searching for and evaluating the source analogues identified; filling data gaps; assessment of the uncertainty associated with the predictions. In particular, the evaluation of source substances validity/suitability covers the similarity assessment between source substance physicochemical profiles as well as the removal of significant outliers. Meanwhile, assessing the metabolic pathway similarity of the source substances can help evaluate commonality in key functional groups, including reactivity as encoded in structural alerts.

The contributions outlined above aim at making practically feasible, as well as more transparent and reproducible, the general provisions provided by guidance documents. The approaches, while overlapping to a certain degree, aim for the same goal of identifying one or more source substances of the target substance. An additional factor of variability is the necessary involvement of expert judgement (Patlewicz et al., 2018). There are a number of commonalities in all approaches, namely the consideration of available data and information across the source substances that is relevant to the endpoint, the incorporation of various types of data and information and the need for expert judgement. Finally, as stated above, it must be acknowledged that all decisions relating to inclusion and exclusion of analogues should be transparent and documented.

A.4 | GENERATING/ASSEMBLING SUPPORTING INFORMATION TO PREDICT THE ENDPOINT(S) OF INTEREST FOR THE TARGET AND SOURCE SUBSTANCE USING NEW APPROACH METHODOLOGIES

Before attempting read-across between a target and source substances, it is advisable that any lacking data relating to physicochemical, toxicokinetic/toxicodynamic aspects, identified in the evaluation of the target/source substance, are filled in as much as possible. The available means for generating/assembling include both non-testing (in silico) and testing (in vitro and in vivo) methods.

The use of in vivo methods is also a last resort possibility, which is subject to a number of constraints due to the regulatory obligations to the 3Rs fundamental principles of replacement, reduction and refinement of animal testing, and the costs, time and ethical issues. This is where the use of NAMs can provide a very useful, cost-effective and ethical means for informing the read-across. It is, therefore, logical that a scheme for assembling/generating should start with the use of NAMs to contribute to strengthen the similarity of source substance and target substance regarding toxicokinetic, toxicodynamic and toxicological characteristics that may support the read-across hypothesis, and in doing so, increase confidence in the read-across assessment. With a view of these aspects, a systematic strategy for such an approach is proposed in **Figure A.1**.

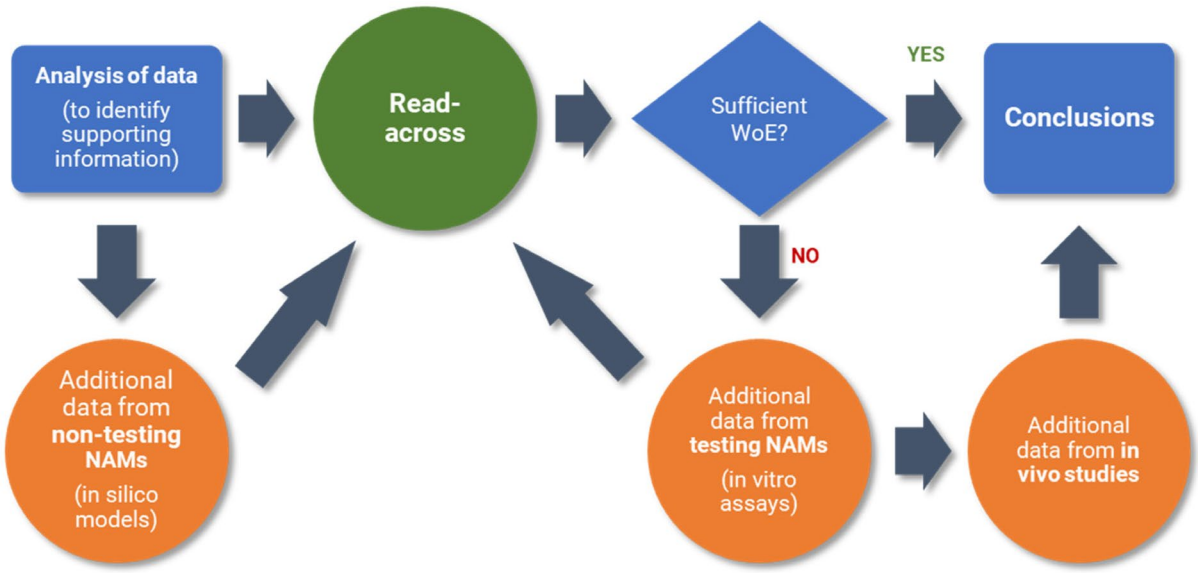

FIGURE A.1 A strategy for generating/assembling supporting information filling for read-across.

Thus, generating supporting information should start with the use of NAMs – where 'non-testing' NAMs, such as in silico quantitative structure-property relationships (QSPR)/QSAR) and physiologically based kinetic (PBK) models, should be used in the first place. The outcome of the in silico assessment may provide supporting evidence for read-across and also help in identifying source analogues for use in the read-across. This additional evidence, together with the other available data identified in the evaluation of the target/source substance steps, should then be evaluated for adequacy to justify a read across. The combined information with the outcome of read-across, may provide sufficient WoE to allow drawing a conclusion on the toxicokinetic, toxicodynamic and/or toxicological similarities between the target and source substances.

Where this combined evidence is weak or contradictory, a recourse to 'testing' NAMs based on relevant in vitro tests should be considered to further strengthen the WoE. Again, the evidence from in vitro tests should be combined with the other information and evaluated to assess whether the WoE is now sufficient to justify a read across. Where the combined information from all of the used NAMs still does not provide a sufficient WoE to support read-across, the use of in vivo tests may be the only last resort to draw a conclusion on the toxicological effect(s) of the target substance.

In this regard, it is noteworthy that NAMs may be useful as additional information to support the read-across hypothesis. As shown in **Appendix B**, the officially validated or scientifically valid in vitro NAMs are largely available for local endpoints (such as skin sensitisation) and genotoxicity. However, chronic and relatively complex endpoints are either not covered or only partially covered, such as repeated dose (sub)chronic toxicity, reproductive toxicity, carcinogenicity and toxicokinetics. Also, while NAMs can provide a good indication for potential endocrine activity, a substance can only be regarded as endocrine active but not as an endocrine disruptor on the basis of these data alone, according to ECHA/EFSA (2018). Supporting information could in such cases still be filled in the context of a relevant AOP together with toxicokinetic modelling. The use of validated NAMs is preferred in risk assessment, although other methods that may not have been formally validated but shown to be scientifically valid may also be considered case by case. In the EU, a formal validation process for alternative methods has been established by the EU Reference Laboratory for alternatives to animal testing and its Scientific Advisory Committee (ESAC). Other organisations that evaluate the validation of alternative methods are the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, National Institute of

Environmental Health Sciences, USA) and the Japanese Center for the Validation of Alternative Methods (JaCVAM, National Institute of Health Science, Japan).

A.4.1 | Tools for read-across

Several tools for read-across have been developed that can assist with, or automate, various steps of the read-across workflow described in **Figure 2**. This section focusses on in silico, or computational, tools that can be applied. The types of tools that can be applied for the various steps in the read-across workflow have the following functionalities or purpose:

- Identification of toxicological (and other) information from databases. As part of the problem formulation, there may be a need to retrieve appropriate identifiers for the source substances from relevant database(s) (Step 1 of the workflow). Compilations of existing data will also need to be searched, to undertake the target substance characterisation (Step 2). An appreciation of existing data will support the problem formulation. Databases will also need to be searched for toxicological information for the analogues during the identification of source substances (Step 3). The same, or different, databases may also contain other pertinent information such as physico-chemical properties or NAM data.
- Identification of structural analogues. Computational tools will enable analogues to the target substance to be identified in an efficient manner (Step 3). Similar substances are normally sought from a database or inventory, of known chemical structures. A variety of techniques to identify similar substances can be applied. Similarity can be assessed by, for instance, the presence or absence of structural features associated with activity (the so-called profilers based on 2D structures), metrics of similarity, etc.
- Estimation of properties. Computational tools may be required to assist in the calculation of physicochemical or toxicokinetics properties (if measured data are not available). Most of these approaches are based around QSAR or QSPR and can be applied in the target substance characterisation (Step 2) and to evaluate the source substances and populate the data matrix (Step 4).
- Reporting. There is a need to capture the information used in the read-across, to form the basis for the justification. This is required to support Step 5 as well as the final conclusion. This could include details on chemical structures and required identifiers, available toxicity data, NAM, TK and property data. It should be noted that an automated report supports, but does not replace, expert opinion with regard to the justification of the read-across argument.

Illustrative examples of in silico tools are noted in **Table A.1**. **It should be noted that the tools listed in this section should neither be taken as a comprehensive list, nor does the listing of a specific in silico model/tool in this guidance document constitute a specific recommendation by EFSA**. Resources are available that provide more comprehensive listings of tools for read-across (Patlewicz et al., 2017), QSARs and QSPRs for toxicity, TK and property prediction (see **Table A.1**), as well as databases (Pawar et al., 2019).

Of the tools described in **Table A.1**, the OECD QSAR Toolbox is freely downloadable and commonly used to support read-across. It remains focused on performing endpoint category and associated read-across and providing a platform to help document and justify on a case-by-case basis the confidence in a read-across by different contexts – starting from formulating categories based on structural similarity or by using MIE information encoded as structural alerts (so-named profilers). Other tools have been more focused on developing objective means of read-across for specific endpoints to facilitate systematic and reproducible predictions. The ToxRead software is publicly available within the VEGAHUB[15]. Other read-across tools developed workflows considering metabolic, structural and biological similarity (Gadaleta et al., 2020), while more recently the VERA software for read-across starts from the chemical category approach (Viganò et al., 2022). VERA and the RAXPY software, implementing the workflow with metabolism, are available within VEGAHUB (Roncaglioni et al., 2022). Other researchers have sought to establish a baseline in read-across performance by systemically comparing how chemical structural information in conjunction with NAM data can be used to predict different in vivo toxicity effects (Low et al., 2013; Shah et al., 2016). The GenRA approach is a tool that implements an algorithmic approach to read-across drawing on machine learning techniques. GenRA is disseminated in two ways – either as a workflow to facilitate a chemical based read-across within the EPA CompTox Dashboard or using a genra-py python library (Helman et al., 2019; Shah et al., 2021).

It should also be noted that tools such as the OECD Toolbox permit a category to be defined based on structural and metabolic characteristics at the same time. In Generalised Read-Across (GenRA), a custom fingerprint can be used to search for analogues based on both biological and chemical fingerprints, whereas Gadaleta's workflow (Gadaleta et al., 2020) permits searches on the basis of biological, structural and metabolic similarity in parallel.

In addition to tools for read across, several commercial systems are also available for the assessment of toxicity of a chemical substance, e.g. QSAR-based systems such SciQSAR® (SciMatics, Inc.) and TopKat® (Toxicity Prediction by Computer Assisted Technology), molecular fragment-based QSAR expert systems such as CASE-Ultra® (Multicase Inc.) and Leadscope® (Leadscope, Inc.) and expert knowledge-based systems such as Derek Nexus® (Lhasa Ltd.).

Some in silico modelling tools are specifically characterised by the capability of deriving structural features and physicochemical properties to be used as part of the process of identifying appropriate source chemicals. Some of these tools, referenced also in **Table A.1**, are described more in detail below.

[15] www.vegahub.eu.

Success has been achieved in identifying moiety/ies automatically by in silico modelling tools – such as SARpy (Ferrari et al., 2013). The fragments obtained from modelling tools may not necessarily overlap with the structural alerts derived by the experts. Compared to the structural alerts, that are derived on the basis of a specific toxicological mechanism, the fragments extracted by modelling tools may only have a statistical significance and may therefore need further consideration from a mechanistic point of view. They are, nevertheless, useful in a read-across for identifying clusters of substances with a specific behaviour. An example software system that has implemented this approach for read-across is ToxRead.[16] Certain physicochemical features can describe the behaviour of the substance that can in turn be interpreted in relation to regulatory endpoints. For example, the role of logP in determining bioaccumulation has been implemented in ToxRead.

Once the fragments specific for the target and the source molecules have been identified, they should be assessed regarding their influence on the adverse effects. The situation is different for endpoints with continuous or categorical values. More difficult is the evaluation of the role of a fragment for a continuous property. For example, a software program that has addressed this is QSARpy, which associates a coefficient with the fragment, to be used to measure the increase or decrease in potency of the effect when a certain fragment is present (Ferrari et al., 2018).

An example of in silico software that implements the structural similarity/dissimilarity approach is ToxDelta[17] (Golbamaki et al., 2017). Another example is VEGA[18] that has implemented several approaches for assessing similarity, as well as dissimilarity and rules for associating activity with physicochemical properties. The approach adopted in VEGA applies different algorithms to evaluate the chemical similarity and then merges the different results into a single tool for similarity. This has been considered a balanced approach after testing with 4 million chemicals (Floris et al., 2014).

A chemical structure may also be defined in terms of physicochemical descriptors – both measured and calculated. There are also several algorithms that can be used to measure similarity between two or more substances on the basis of such descriptors, and to identify source chemicals. Among these is the kNN algorithm, a non-parametric statistical method that relies on the similarity between analogues. It does not contain any information on the mechanism (as in the expert systems), on the role of the descriptors (as in the QSAR models), or on the toxicity (which is the output of the model). kNN is performing well when there are very similar substances and fails when similarity is low. Furthermore, kNN is performing well if toxicity label of the similar substances is homogeneous, but it cannot resolve any conflicts.

An example of software system that has several kNN tools implemented is VEGA (Manganaro et al., 2016). An agreement on the property values between the most similar substances increases the confidence in the prediction, and this is evaluated by the system, providing a measurement of the reliability. Analogue similarity can be also assessed with neural network based systems – some of which are related to kNN.

The structural similarity assessed through kNN can also be combined with toxicological information and some physicochemical properties. For instance, ToxRead integrates different levels of information, combining different kinds of kNN. The first level of kNN assessment is done based on the structural similarity, but the software also searches for the most similar substances sharing the same toxicological profile that is represented by the fragments associated with the adverse effect(s), or structural alert(s). If there is one or more structural alert within the target substance, the software provides all the most similar substances (up to 100) with that structural alert, with the possible alerts visualised in a graphical format. This level of information is endpoint specific and, therefore, there are many modules for read-across within ToxRead, for the individual endpoints.

Another comprehensive approach based on using all the information derived from chemical structure and other sources, has been implemented in the UBA-toDIVINE software.[19] UBA-toDIVINE software is aimed at providing a single programme integrating the results of in silico models and read-across. For read-across, several metrics for similarities have been developed and integrated, based on chemical, toxicological, physicochemical and toxicokinetic and transformation aspects. For the chemical similarity, the components are similar to those implemented in VEGA and some of the properties used are either based on experimental values or calculated by VEGA. For toxicodynamic similarity, the main components relate to the presence of structural alerts or the MoA. For toxicokinetics and transformation products, the key components are half-life and a collection of more than 200 environmental transformation products, to evaluate the similarity of the target substance and its degradation products to those of the source substances. Each of the four similarity metrics (individually composed by several components) are then integrated into a single overall similarity score through assigning weights to each metric. This way, the software measures the overall distance between the target substance and the similar substances. This software is property of the German UBA and is currently restricted because it contains property values of REACH registered substances.

[16] https://www.vegahub.eu/portfolio-item/toxread/.
[17] https://www.vegahub.eu/portfolio-item/toxdelta/.
[18] https://www.vegahub.eu/.
[19] https://chm.kode-solutions.net/pf/uba-todivine/.

TABLE A.1 A non-exhaustive summary of freely available in silico tools and resources to support grouping and read-across.

Computational tool	Description and functionality	Source	Steps in read-across workflow
OECD QSAR Toolbox	Read-across tool allowing for chemical identification, profiling and similarity estimation for analogue identification, data gathering and report generation.	https://qsartoolbox.org/ (Dimitrov et al., 2016; Schultz et al., 2018)	Steps 2–5
ToxRead	Displays similar chemicals and also the structural alerts and relevant features in common between chemicals.	https://www.vegahub.eu/portfolio-item/toxread/	Steps 2–4
VERA	Assessment of the similarity between chemicals using structural alerts specific to the property, predefined molecular groups and structural similarity.	https://www.vegahub.eu/portfolio-item/vera/	Steps 2–6
RAXPY	A read-across tool based on structural, biological and metabolic similarities.	https://www.vegahub.eu/portfolio-item/raxpy/	Steps 2–4
Generalized Read-Across (GenRA)	An algorithmic approach to permit objective and reproducible read-across predictions of toxicity and bioactivity.	https://comptox.epa.gov/genra/; https://www.epa.gov/comptox-tools/generalized-read-across-genra (Helman et al., 2019; Shah et al., 2021)	Steps 2–3
Toxicity Estimation Software Tool (T.E.S.T.)	QSAR models to predict a variety of toxicological and other endpoints.	https://www.epa.gov/comptox-tools/toxicity-estimation-software-tool-test	Step 2
Hazard Evaluation Support System Integrated Platform (HESS)	Supports, including access to a database, the evaluation of repeated dose toxicity by category formation.	https://www.nite.go.jp/en/chem/qsar/hess-e.html	Step 2
LRI AMBIT Read across	Consists of a MySQL database and functional modules supporting queries, data mining, and building and application of predictive model.	https://ambit.sourceforge.net/	Steps 2–3
Danish QSAR database	A tool that allows users to search for hazard information on chemical substances, especially those with little or no testing data.	https://qsar.food.dtu.dk/	Steps 2–3

Available in vitro methods

The development and validation of in vitro methods is a dynamic process, and a number of resources are available for further information on the available NAMs. Also, EFSA hosts supporting tools for NAMs e.g. TKPlate, the open-access platform for toxicokinetic and toxicodynamic modelling (Dorne et al., 2023) and has also reviewed the use of NAMs (for the application in risk assessment of Nanoparticles) in the food and feed sector (Usmani et al., 2024). Furthermore, because of the EU ban on animal testing of cosmetic ingredients, the use of NAMs has become critically important for assessing safety of the chemicals used as cosmetic ingredients. The Scientific Committee on Consumer Safety (SCCS) has recently compiled a list of the available in vitro NAMs in their guidance documents (SCCS/1647/22 and SCCS/1655/23 (SCCS, 2023b, 2023a)), a summary of which is provided in **Appendix B**. This is expected to be continually updated as more methods reach the status of regulatory readiness.

Adverse outcome pathways

The use of AOP information potentially can provide valuable support for read-across. All OECD endorsed AOPs can be found at the OECD website.[20] The AOP wiki[21] in addition hosts AOPs undergoing OECD review, as well as AOPs under development. Moreover, under the auspices of the OECD integrated approaches to testing and assessment (IATA) project, several AOP-supported read-across case studies reviewed by the OECD members are published, as well as consideration documents on the learnings and lessons from the review experience.[22]

Additional information on case studies is provided **Appendix D**.

A.5 | USE OF NEW APPROACH METHODOLOGIES (NAMS) TO SUPPORT READ-ACROSS

As described above, the objective of NAMs integration into read-across is to contribute to ascertain the similarity of the source substance(s) and target substance relating to toxicokinetic and toxicodynamic characteristics, and in doing so, increase confidence in the read-across assessment. The following section outlines how NAMs data can be assembled/generated to inform/reduce the uncertainty of the read-across hypothesis. It is to a large extent based on the approach developed in the EU-ToxRisk project (Escher et al., 2019).

A.5.1 | New approach methodologies supporting toxicodynamic similarity

A commonly encountered scenario for chemicals identified as category members is that more than one relevant toxicological effects may have been observed in an in vivo study. The intention of a read-across assessment is to describe all these effects and identify which are considered critical lead effects that need to be tested. Effects observed at higher dose only, i.e. being less potent than the lead effect, can be left out from testing, but would need justification. For instance, the absence of a trend for such an effect could be an argument. If this higher dose toxicity only concerns general toxicity (e.g. body weight changes), then additional testing for this as part of the read-across approach might not be needed. It is noted that the selection of specific experimental assays in the different scenarios described below may be based on their representation of typical apical readouts or readouts representing KEs or MIEs (see also **Table B.1**). Overall, three different situations can occur, which require different data assembling/generation strategies.

Situation 1: Target and source substances share a known AOP/MoA

A known and shared AOP or MoA for the lead effect can guide a targeted data generation/assembly. When the AOP is OECD endorsed, appropriate assays may have been already identified, facilitating the design of a testing/data assembling strategy and reducing the uncertainty of this part of the assessment.

If the AOP is not yet endorsed by the OECD, the selection of assays needs to be well justified and described, providing biologically plausible evidence that the assays/data cover the relevant MIE and/or involved KEs. Information on the specificity of the assay for a given MIE/KE, robustness of the assay (is it repeatable and reproducible) and assay response characteristics is needed.

Data/testing from late KEs (close to the adverse outcome) provides more confidence than data/testing from MIEs and early KEs (close to the MIE), since the latter may not culminate into late KEs and thus, not cause the adverse outcome under evaluation. Confirmation of appropriate testing/data is obtained when substances, which produce the lead effect in vivo, do also activate the MIE and KEs in the selected in vitro assays. In case source substances show a potency trend for the induced lead effect, an in vivo negative source substance could function as true negative of the in vitro model(s), thus confirming the performance of the chosen assays. Not all MIEs and KEs need to be tested: a justified selection of MIEs and KEs from the AOP/AOP network enabling the evaluation of the biological profile should be sufficient to identify critical effects. In case the read-across involves more than one AOP, testing/data assembling strategies per AOP should be developed and transparently described.

[20] https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x.

[21] https://aopwiki.org/.

[22] https://www.oecd.org/chemicalsafety/risk-assessment/iata/.

Situation 2: Shared specific effects, AOP or MoA unknown

If the AOP or MoA for the specific lead effect is not known, the selection of appropriate assays is only steered by knowledge of the involved target organ(s) in vivo. However, a case for a targeted data generation/assembly can still be justified. The selection of assays/data needs to be well-justified and described, providing biologically plausible evidence that the assays/data do cover the relevant lead effect observed in vivo. If the in vivo effect can directly be monitored in cell cultures (e.g. lipid accumulation in hepatocytes), this readout should be monitored on that cellular level. If the in vivo effect is less specific and/or has multiple MoAs, assays that have broad coverage of endpoint toxicity mechanisms should be included and monitored to verify whether the source substances and target substance have effect patterns (profiles) supporting their category membership with regard to the lead effect.

In case the lead effect endpoint concerns development and reproduction, a battery of tests that collectively have a broad coverage of applicable endpoint toxicity mechanisms might be applied. All endpoints need to be followed for establishing category membership based on effect patterns. The developmental neurotoxicity testing battery and adhering guidance (OECD, 2023a) is an example of such an in vitro battery, however, for the time being a battery of assays with a wide coverage of development and reproductive endpoints has not been established. In case the read-across involves more than one effect, testing strategies per effect should be developed and transparently described. As for scenario 1, fit-for-purpose assembled/generated data should be ascertained by information on specificity, robustness and response characteristics.

Situation 3: Shared non-specific toxicity/low toxicity

If the common critical lead effect of selected source substances is a non-specific adverse effect, like a significant decrease in body weight or changes in (relative) organ weights in the absence of, e.g. a more specific histopathological change or changes in ancillary findings, the selection of appropriate assays/data assembly must follow an untargeted approach (a wide spectrum of data assembly/generation). Currently, for practical purposes, such an approach remains to be presented as it is not clear what biological pathways need to be covered by such a screening approach to claim a similar non-specific effect like body weight change. However, in applying battery approaches in the future, all endpoints need to be considered for establishing category membership based on effect patterns. Again, in case the read-across involves more than one lead effect, testing strategies per effect should be developed and transparently described. As for the previous situations, fit-for-purpose assembled/generated data should be ascertained by information on specificity, robustness and response characteristics.

Predicting low toxicity

If the selected source substances hardly show in vivo toxic effects, or no clear toxicity at all, first the cause of the absence of effects should be investigated. For instance, can it be explained by toxicokinetics?

For establishing read-across for substances of low toxicity, it is especially important to identify relevant assays for the purpose as well as establishing the performance of the assays used, with appropriate concurrent positive controls (these substances are not necessarily part of the read-across, but the data should be presented).

In all three situations, not only hazard identification but also hazard characterisation can be part of problem formulation. Once identifying the intrinsic toxicological similarities, the applied tests can also deliver information on the potency of substances. To benchmark to regulatory relevant top-dose (usually 1000 mg/kg), in vitro testing should correspond to such a dose established by reverse dosimetry (see below).

A.5.2 | New approach methodologies supporting toxicokinetic similarity and exposure consideration

An understanding of ADME properties in vivo is important to identify and explain (dis)similarities and trends between target and source substances (e.g. systemic bioavailability, common active metabolites). However, often in vivo toxicokinetic data are not available. QSAR/QSPR models can predict ADME parameters such as the fraction absorbed (f_a) of the external exposure dose, the fraction unbound in plasma (f_u), tissue to plasma partition coefficients ($K_{p,t}$) and steady-state volume of distribution (V_{ss}; L/kg bw). Other parameters, such as intrinsic hepatic clearance ($CL_{\text{int,hep}}$) lack robust QSAR models at the moment, and can be determined through in vitro assays (e.g. in primary hepatocytes, liver microsomes).

Although such parameters give an insight into similarities and trends among substances, PBK modelling can be used to predict bioavailability and systemic/tissue exposures (i.e. concentration-time profiles) in humans and model organisms. By in vitro to in vivo extrapolation (IVIVE) coupled PBK models, substance properties (derived from QSAR and in vitro assays) are integrated with physiological parameters. For example, intrinsic hepatic clearance rate measured in vitro in human liver microsomes needs to be extrapolated to estimate in vivo whole-liver human hepatic clearance rate. Thus, in a read-across context, developing the IVIVE-PBK models can support the comparison between the target substance and source substance in terms of systemic and target organ exposures.

To contextualise in vitro toxicodynamic concentrations to in vivo exposure, there is the need to perform quantitative IVIVE (QIVIVE), which requires an assessment of in vitro toxicokinetics. However, in most in vitro assays, the dose is estimated as the nominal media concentration. The relationship between the media concentration and cellular or target concentration is influenced by several parameters, e.g binding to media serum proteins and/or the plastics of cell plates,

active versus passive uptake into cells, cell density and the degree of any metabolism and parent substance stability in the culture (Paini et al., 2019). Models have been developed for predicting the in vitro distribution to, e.g. cells, serum constituents (Proença et al., 2019).

Guidance on documenting and reporting of PBK modelling and simulation is provided in OECD guidance document (OECD, 2021), but there is a lack of internationally accepted guidance on QIVIVE procedures and models (OECD, 2023a). Critical in the context of read-across is the need to justify the assumptions made in the PBK modelling approach applied across all the source substances and target substance. Also, the same model framework and underlying assumptions should be applied for all source substances and target substances to avoid bias.

A.6 | HOW TO CHARACTERISE AND ADDRESS UNCERTAINTY IN READ-ACROSS

A.6.1 | Context and purpose

As discussed above, read-across is a multistep process, each of which may be associated with a certain level of uncertainty. Therefore, the identification and characterisation of uncertainty associated with each aspect of a read across is crucial for justification of the approach. In a general sense, the consideration of uncertainty may relate to any aspect of the read-across that will introduce variability and/or bias, with reference being made to characterisation of uncertainty as described by EFSA (EFSA Scientific Committtee, 2018a). The present guidance has taken a broad definition of uncertainty to include any element of the read-across that is associated with uncertainty, variability or may be subject to bias. The process of assessing uncertainties is described by EFSA and involves the identification of uncertainties, their characterisation and, where possible, quantification. It is the intention of this guidance to identify all relevant uncertainties and provide practical illustration of how a user may characterise and (semi-)quantify the identified uncertainties (see **Chapter 4**). The purpose is to provide an overall statement on uncertainty. Furthermore, this guidance sets forth a standardised protocol for read-across, which aims to define how low uncertainty could be achieved.

A.6.2 | Background to the assessment of the uncertainty of a read-across

Assessment of uncertainty is performed within Step 6 of the read-across workflow, but relates to every aspect of the read-across and should also be incorporated into the problem formulation. It is envisioned that a framework to assess uncertainty will be utilised and is presented in Chapter 4. The framework is designed to identify all relevant uncertainties and provide a means for the user to characterise them. Currently, there is no agreed framework for assessment of uncertainty of a read-across, and it is largely based on expert opinion and interpretation. This guidance is therefore intended to provide principles for proper and consistent application of uncertainty analysis in read-across.

There have been many attempts to define a strategy that describes, and to some extent quantifies, the uncertainties of a read-across, notably from (Blackburn & Stuard, 2014; Schultz et al., 2022; Wu et al., 2010). Definitions of uncertainty have also been provided in other publications (e.g. (EFSA Scientific Committee, 2018a, 2018b; WHO-IPCS, 2004, 2018), as well as the definition and application of uncertainty in terms of modelling studies (Brozek et al., 2021). The value of other approaches to assist in the identification and characterisation of uncertainties is also recognised, notably in ECHA's RAAF (ECHA, 2017c), and the contribution from the EU-ToxRisk Project (Escher et al., 2022).

Building on from different concepts (Blackburn & Stuard, 2014; ECHA, 2017c; Wang et al., 2012; Wu et al., 2010), Schultz et al. (2018)) proposed a unified and harmonised approach to define uncertainties and identified 12 components of uncertainty for read-across in four main themes. These comprise the uncertainty related to regulatory use, the data to be read-across, the argumentation and the justification of similarity, all of which are crucial to be characterised. Each type of uncertainty was associated with one or more questions to assist the developer/reviewer to characterise and (semi-)quantify the uncertainty.

A.6.3 | Characterising uncertainty in a read-across

Given the uncertainties identified in the read-across, for instance as listed in **Table C.1**, characterisation of such uncertainties is required. The process of characterising uncertainty is intended to provide a documented analysis and assessment of each identified uncertainty, with the guidance in Chapter 4 providing a series of questions and illustrative responses. Documentation of uncertainty will be possible with a template found in **Appendix C**.

The characteristics of uncertainty may be quantified. However, compared to the identification of uncertainty, the quantification is generally not straightforward, and only semi-quantification may be possible in most cases. (Schultz et al., 2015) provided an assessment of various methods to quantify uncertainty, concluding that there are three possible approaches:

- a sliding (continuous) scale;
- a weighted scale;
- the use of predefined divisions (e.g. qualitative assessment, which is unweighted).

The ECHA RAAF provides another means of assessing most of these elements. It appears that a pragmatic solution, at the current time, is to assess uncertainty in a categorical manner – with three categories (low, moderate and high uncertainty).

This, however, requires unambiguous, clear and accountable definition of these uncertainty categories for a consistent application in read-across. In this regard, Pestana et al. (2021)) provided the following definitions, which give a starting point for assessing uncertainty of read-across:

- Low Uncertainty: Strong or compelling evidence that the molecules are similar with regard to the criterion being assessed as related to the defined toxicity or adverse effect, e.g. demonstrable similarity from relevant or pertinent experimental data (preferably) or in silico predictions.
- Moderate Uncertainty: Partial evidence that molecules are similar with regard to the criterion being assessed as related to the defined toxicity or adverse effect, e.g. some demonstrable similarity from experimental or in silico data. Some experimental data may be missing or from non-standard or only related tests.
- High Uncertainly: No or very little evidence that molecules are similar with regard to the criterion being assessed as related to the defined toxicity or adverse effect e.g. no or very limited experimental data and/or no consideration of in silico predictions.

The use of categorical approach to uncertainty assessment, while not providing full quantification, is a simple means of identifying areas of the read-across where uncertainty may not be acceptable. The purpose here would be to reduce associated uncertainty through the inclusion of further data and/or analysis. An obvious example of a means to reduce uncertainty is the inclusion of targeted NAM data.

A.7 | READ-ACROSS DOCUMENTATION

There have been several different attempts at structuring how read-across could be documented to help describe the rationale of the category/analogue approach and how a read-across prediction is being performed for a specific endpoint.

A matrix of data availability should be constructed for the target endpoint and all other relevant endpoints. The matrix should include the chemical of interest (target substance) and (source substance(s). If multiple analogues are identified, they should be arranged in a suitable order. The ordering should reflect a trend or progression within the group. The cells of the matrix should indicate whether data are available or unavailable (*adapted from* (OECD, 2014a)). The cells of the matrix should contain the chemical identifiers, a physicochemical description of the chemicals with properties of general relevance, especially for bio-availability (e.g. logP, molecular weight, water solubility, logD, pKa), and the available reliable key study results. In compliance with these basic requirements, different notable contributions and proposals should be mentioned. These approaches reflect an evolving scenario characterised by the different regulatory needs and expanding scientific progress, together with the perceived need for harmonisation of templates.

A first example is represented by the report template included in the ECHA guidance on (Q)SAR and Read Across (ECHA, 2008). These schemes have been implemented into the OECD QSAR Toolbox. The OECD (Q)SAR Toolbox[23] is a standalone free software application developed under the coordination of OECD and ECHA. It was designed with the purpose of facilitating practical application of QSAR approaches within regulatory frameworks. Crucial to the workflow that culminates in the data gap filling, is the grouping of chemicals into chemical categories by read-across (Dimitrov et al., 2016). The line of reasoning in the Toolbox applications combines structural/chemical and mechanistic considerations. The chemical, biological (e.g. ToxCast outcomes) and toxicological data to be used are contained in a large range of databases embedded in the Toolbox. Customised databases can be easily uploaded and integrated into the Toolbox by the user. Users of the QSAR Toolbox can automatically generate two documents that present the data and illustrate the whole process: a prediction report and a data matrix. There is also an option to generate a read-across justification document based on RAAF Assessments Elements which may be relevant in some regulatory contexts.

A second source of templates are those developed by OECD in the context of its IATA project. As a part of IATA, the OECD has been developing guidance documents and tools for the use of alternative methods such as (Q)SARs, chemical categories and AOPs. In particular, the OECD developed guidance on the AOPs concept that supports the development of mechanism-based NAMs.

There is a need for the investigation of the practical applicability of these methods/tools for different aspects of regulatory decision-making, and to build upon case studies and assessment experience across jurisdictions (OECD, 2022). Part of this investigation is the development of a reporting template that can accommodate complex NAMs data: A template is reported in the above-mentioned OECD document. The IATA template is characterised by high flexibility regarding the type of data accepted, that must be inserted manually.

As an example, van der Stel et al. (2021) generated two case studies, and applied a range of NAMs to explore the toxicodynamic properties of the substances, e.g. in silico docking, as well as in vitro assays and readouts, including transcriptomics, in various cell systems, all anchored to the relevant AOPs. The data matrix follows the structure of the original IATA documents and is subdivided into 'purpose' and 'analogue information'. Purpose depicts the scope of both read-across case studies, the included substances (source and target) and the read-across approach. Source substance information describes chemical-specific information concerning physical/chemical properties, in vivo ADME, the coupled AOPs, and other relevant information collected from external sources.

[23] https://qsartoolbox.org/.

An important source of information for data matrices lies in submissions to IUCLID 6 (International Uniform Chemical Information Database). This is a software developed by ECHA in cooperation with the OECD to record, store, maintain and exchange data on intrinsic and hazard properties of chemical substances. It has become a key software in IT environments of organisations that manage scientific data on chemicals in a regulatory context, e.g. OECD HPV, EU Biocides, EU REACH[24] and EU pesticides.[25] It is the reference implementation of the OECD Harmonized Templates (OHT), which are standardised data formats agreed by OECD members that can accommodate studies and other information (e.g. use of grouping and read-across) on chemicals to determine properties or effects on human health and the environment, as well as data on use and exposure. Whereas IUCLID 6 is a mature tool for storing and exchanging information on substances (e.g. for REACH applications), automated generation of data matrices would require repurposing of the IUCLID extraction tools or functionalities according to organisational needs. General information on IUCLID 6, and how to report read-across is in ECHA submission manual.[26]

Furthermore, Blackburn and Stuard (2014) assessment framework proposed a template to structure considerations for analogue evaluation – this was organised like a decision workflow/questionnaire to guide the relevant considerations and allow for a systematic evaluation of the relevance and suitability of candidate analogues. (Schultz et al., 2015) embedded elements of the above mentioned uncertainty framework (Blackburn & Stuard, 2014) but proposed many additional templates to structure how different data matrices could be constructed to facilitate the side-by-side comparison of category members with respect to the different similarity contexts, e.g. a template for comparing physicochemical properties, a template to compare structural alerts and reactive groups between members, a template to compare KEs in an AOP, etc. Outcomes from these individual templates could then be summarised in an overarching uncertainty template to document the uncertainty in the data for each of these contexts, as well as the strength of evidence in each case in order to determine an overall uncertainty in the similarity of the category members and the impact this would have on the associated read-across predictions. As seminal in its efforts to characterise how evidence could be structured to help strengthen a category justification, there were two potential limitations to the approach. Firstly, the assessment was qualitative and subjective, a fact which is inherent in the read-across approach as is practiced so far and arguably a reason why acceptance is still thwarted since there are no objective benchmarks to assess performance. Secondly as novel data streams are accessible and are large in size, e.g. phenotypic profiling with 1300 features or transcriptomic data with many thousands of features – summarising and extracting insights of how this type of data strengthens a category approach does not readily lend itself to a qualitative assessment.

[24]Regulation (EC) No 1907/2006 - Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). https://eur-lex.europa.eu/eli/reg/2006/1907.

[25]Regulation (EC) No 1107/2009 and Regulation (EC) No 396/2005.

[26]ECHA (October 2023) How to prepare registration and PPORD dossiers https://echa.europa.eu/documents/10162/22308542/manual_regis_and_ppord_en.pdf/891754cb-a6b6-4bb6-8538-52ccde74070e.

APPENDIX B

In vitro methods for toxicological characterisation of chemical substances

TABLE B.1 Examples of in vitro methods for toxicological characterisation of chemical substances, comprising representative (formally validated and scientifically valid) in vitro methods.

Data required for risk assessment		In vitro methods*
Toxicokinetic aspects	ADME data to indicate bioavailability of the target substance, or possible bioaccumulation	Skin absorption in vitro [OECD TG 428 (2004)].
Toxicological aspects	Acute toxicity	The only validated in vitro method at present for acute oral toxicity (EURL ECVAM endorsed) is the 3T3 NR (Neutral Red) uptake test, applicable for non-classified chemicals, based on a cut-off of LD50 > 2000 mg/kg bw (EC JRC, 2013). EURL ECVAM has issued recommendations concerning the validity and limitations of this in vitro test (EC JRC, 2013). An OECD acute toxicity waiver guidance document (OECD, 2017c) includes, among other criteria, the possibility to waive the acute oral toxicity study based on the results of an alternative test or test battery, if the LD50 is predicted to be greater than 2000 mg/kg.
	Skin and eye irritation	**Skin corrosion:** a. Rat Skin Transcutaneous Electrical Resistance (TER) test [OECD TG 430 (2015)] b. EpiSkin™ [EC B.40bis, OECD TG 431 (2004)] c. EpiDerm™ SCT (EPI-200) [EC B.40bis, OECD TG 431 (2004)] d. SkinEthic™ Reconstructed Human Epidermis (RHE) [EC B.40bis, OECD TG 431 (2004)] e. epiCS® (former Epidermal Skin Test-1000) [EC B.40bis, OECD TG 431 (2004)] f. The In vitro Membrane Barrier Test Method [OECD TG 435 (2015)] currently only includes the Corrositex™ test. **Skin irritation**: The OECD TG 439 (2021) is stand-alone replacement test within a WoE approach [EC B.46]. The available in vitro models for skin irritation include: a. EpiSkin™ b. EpiDerm™ SIT (EPI-200) c. SkinEthic™ RHE d. LabCyte EPI-MODEL24 SIT e. epiCS® f. Skin+® g. KeraSkin An OECD guidance document is available on an Integrated Approach on Testing and Assessment (IATA) for Skin Corrosion and Irritation (OECD 2017b). **Serious eye damage and eye irritation** Dermal irritancy or corrosivity data should be considered as a first step [OECD TG 439 (2021)]. Several in vitro test guidelines are available to address eye irritation or serious eye damage: a. Bovine Cornea Opacity Permeability (BCOP) test method [OECD TG 437:2020] b. Isolated Chicken Eye (ICE) test method [OECD TG 438:2018] c. Short Time Exposure (STE) test method OECD TG 491 (2020)] d. Fluorescein Leakage (FL) test [OECD TG 460 (2017)] e. Reconstructed Human Cornea-like Epithelium (RhCE) test method [OECD TG 492 (2019); TG 492B (2024)] f. Vitrigel® - Eye Irritancy Test [OECD TG 494 (2021] g. In vitro Macromolecular Test [OECD TG 496 (2019)] h. Defined Approaches for Serious Eye Damage and Eye Irritation [OECD TG 467 (2022)]

(Continues)

TABLE B.1 (Continued)

Data required for risk assessment	In vitro methods*
Skin sensitisation	A number of validated in chemico/in vitro tests are available for skin sensitisation: In chemico skin sensitisation: An OECD Key Event-Based Test Guideline is available for in chemico skin sensitisation assays addressing the AOP Key Event on Covalent Binding to Proteins [OECD TG 442C (2022)] describing: – The Direct Peptide Reactivity Assay (DPRA) – The Amino acid Derivative Assay (ADRA) – The kinetic Direct Peptide Reactivity Assay (kDPRA) In vitro activation of keratinocytes: – ARE-Nrf2 Luciferase Test Method OECD TG 442D (OECD 2022f) describing • ARE-Nrf2 luciferase KeratinoSens™ test method • ARE-Nrf2 luciferase LuSens test method – In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the AOP for Skin Sensitisation OECD TG 442E (OECD 2022g) describing the: • Human Cell Line Activation test (h-CLAT) • U937 cell line activation Test (U-SENS™) • Interleukin-8 Reporter Gene Assay (IL-8 Luc assay) • Genomic Allergen Rapid Detection (GARD™) for assessment of skin sensitisers (GARD™skin) An OECD technical guideline is available for Defined Approaches for skin sensitisation [OECD TG 497 (2021)]. An OECD guidance document is available for the OECD Guideline 497 on Defined Approaches for Skin Sensitisation [OECD GD 336 (2021)].
Short-term toxicity (90-day study in two species)	Currently there is no validated or generally accepted alternative method.
Mutagenicity/ Genotoxicity (in vitro and in vivo)	Base level testing consists of the following in vitro 2-test battery: 1. Bacterial reverse mutation test [OECD TG 471 (1997] for gene mutation testing 2. In vitro Micronucleus test [OECD TG 487 (2016)] for both structural (clastogenicity) and numerical (aneugenicity) chromosome aberrations testing Other in vitro genotoxicity test methods: – In vitro mammalian cell gene mutation tests using the Hprt and xprt genes [OECD TG 476 (2016)] – In vitro mammalian cell gene mutation tests using the thymidine kinase gene [OECD TG 490 (2016)] – In vitro mammalian chromosome aberration test [OECD TG 473 (2016)] Supportive tests in overall WoE approach (for mechanistic understanding): – Pig-a test in vitro (mutation of glycosylphosphatidylinositol (GPI) anchor proteins on the cell surface) – in vitro Comet assay for detection of strand breaks; – in vitro Comet assay modified with lesion specific repair enzyme for detection of oxidation lesions (oxidised purines and pyrimidines) – toxicogenomics (genes involved in DNA instability) – recombinant cell models (GreenScreen HC, BlueScreen HC, ToxTracker) – γH2AX – epigenetic responses (e.g. DNA methylation, non-coding small single-stranded RNAs termed microRNAs (miRNAs) and histone modifications)
Reproductive toxicity	No validated alternative method is available for the assessment of this complex endpoint, and various stages are not likely to be mimicked by a single alternative method (Marx-Stoelting et al., 2009; SCCS, 2023b). Three alternative methods have been validated for embryotoxicity, but not accepted for regulatory use. They were not specific enough to show embryotoxicity: 1. The Whole Embryo Culture test (WEC) 2. The MicroMass test (MM) 3. The Embryonic Stem cell Test (EST) [ESAC 2001].

TABLE B.1 (Continued)

Data required for risk assessment	In vitro methods*
Carcinogenicity	No validated alternative in vitro method is available as OECD test Guidelines to determine the carcinogenic potential of a chemical substance. Some in vitro approaches, such as toxicogenomics, in an overall WoE approach may be useful in indicating the potential carcinogenicity of mutagenic/genotoxic as well as non-genotoxic substances. The conclusion on the carcinogenic potential of mutagenic or genotoxic substances may be made based on the outcome of in vitro mutagenicity tests. A positive in vitro result in mutagenicity testing is seen as indicative of the carcinogenic potential of substances (SCCS/1647/22). In this regard, cell transformation assays (CTAs) can be very useful in identifying mutagenic/genotoxic and non-genotoxic carcinogens, especially when used in combination with toxicogenomics data in a weight of evidence approach. Where a structural alert for carcinogenicity is present, or positive results are obtained in an in vitro mutagenicity tests, the following cell transformation tests may be needed: – an in vitro Syrian Hamster Embryo (SHE) Transformation Test [OECD guidance document 214 (2015)] (CTA). – an in vitro Bhas 42 assay [OECD guidance document 231 (2016)] (CTA) In addition, some information on the carcinogenicity potential can be inferred from mechanistic studies, e.g. on cell proliferation, altered gap junction intercellular communication (GJIC) (Spannbrucker et al., 2019), hormone- or other receptor binding, immunosuppressive activity (Huaux, 2018), ability to inhibit or induce apoptosis, or ability to stimulate angiogenesis or the secretion of angiogenesis factors (Medina-Reyes et al., 2019).
Endocrine activity	A number of in vitro assays are available to indicate endocrine activity: • Oestrogen [OECD TG 493 (2015), US EPA TG OPPTS 890.1250] or androgen receptor binding affinity (US EPA TG OPPTS 890.1150, 2009) • Oestrogen receptor transactivation [OECD TG 455, (2021), US EPA TG OPPTS 890.1300], human cell-based reporter gene assay (ISO 19040-3:2018), yeast oestrogen screen (ISO 19040-1, 19040-2:2018) • Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals [OECD TG 458 (2020) • Steroidogenesis in vitro [OECD TG 456 (2022), US EPA TG OPPTS 890.1550, 2009] • Aromatase Assay (US EPA TG OPPTS 890.1200) • Thyroid disruption assays (e.g. thyroperoxidase inhibition, transthyretin binding). A project on validation of selected in vitro methods within EU-NETVAL activity is ongoing. • Retinoid receptor transactivation assays • Other hormone receptors assays as appropriate High-Throughput Screens, See OECD GD No. 211 (2014) Describing Non-Guideline In vitro.
Immunotoxicity	Currently, there are no regulatory documents specifically dedicated to evaluating immunotoxicity and assessment is performed based on existing guidelines for medicinal products. The available in vitro assays that can also be regarded as broadly predictive of the functional alterations of the immune system, including the Colony Forming Unit-Granulocyte Macrophage assay, the leukocyte proliferation test (immunomodulatory assays), and platelet aggregation, leukocyte procoagulant activity, and various plasma coagulation tests (thrombogenicity assays).

*OECD TG mentioned in the Table, unless specified, are available at OECD Guidelines for the Testing of Chemicals from https://www.oecd.org/en/topics/sub-issues/testing-of-chemicals/test-guidelines.html (OECD, n.d.).

APPENDIX C

Template for uncertainty assessment in read-across

TABLE C.1 Template for uncertainty assessment in read-across.

Uncertainty	Evidence	Assessment of uncertainty (high/moderate/low)	Questions/interpretation	Criteria for low uncertainty relating to a standardised procedure
Definition of the purpose of read-across		H/M/L	Is the intention of the read-across unambiguously defined in terms of e.g. substance, endpoint, use etc.	Clear statement of purpose of read-across
Unambiguous description of chemical structure/substance identity		H/M/L	Is the substance identified and defined? For a single chemical structure, is an unambiguous structure provided including all stereochemistry, salts etc.?	Single substance defined with minimum of IUPAC name, CAS Number, SMILES, InChi
Statement of impurities		H/M/L	Are potential impurities in the substance known and identified?	Statement of impurities above 0.1%
Identification of existing data for target substance		H/M/L	Have all relevant publicly available toxicity data for the endpoint been provided?	Searching of relevant databases, e.g. OECD QSAR Toolbox, ECHA Database etc.
Identification of data gap to be filled i.e. single endpoint or full toxicity profile		H/M/L	Has the endpoint to be filled been stated?	Statement of the endpoint to be read-across
Knowledge of the MoA		H/M/L	Is there a defined MoA that can account for toxicity?	Defined MoA. Note: May not be possible for low or unspecific toxicity substances
Availability of an AOP (and level of completeness)		H/M/L	Is there an AOP to support the MoA. Is it endorsed? How complete is it?	Robust AOP available. Note: Very few endorsed AOPs are available.
Suitability of MoA to support read-across		H/M/L	Does the MIE have a structural basis, is this related to similarity, are there assays and data to provide weight of evidence?	Mechanism supports read-across
Definition of similarity rationale		H/M/L	Is similarity fully described and justified?	Full justification of similarity encompassing relevant aspects for the read-across, e.g. chemical structure, properties, anticipated TD and TK similarity etc.
Quality of databases searched to find analogues		H/M/L	Which databases were searched to find suitable analogues. Were all compilation searched?	High quality and extensive databases searched for analogues.
Definition of similarities and dissimilarities between target and source structures		H/M/L	Are appropriate similarities and dissimilarities between target and source molecule(s) defined and quantified, where necessary including structural features and physicochemical properties?	Full definition of structural and molecular similarities and dissimilarities
Definition of TK similarity		H/M/L	Is TK similarity with supporting evidence provided?	Evidence of TK similarity

TABLE C.1 (Continued)

Uncertainty	Evidence	Assessment of uncertainty (high/moderate/low)	Questions/interpretation	Criteria for low uncertainty relating to a standardised procedure
Definition of TD similarity		H/M/L	Is TD similarity with supporting evidence provided?	Evidence of TD similarity
Data quality (including for supporting data)		H/M/L	Are toxicity data to be read across high quality and appropriate for the endpoint?	High quality and relevant data used for read-across
Overall WoE established		H/M/L	Is there consistency in all data and supporting evidence?	Multiple lines of evidence for the target and source substance(s) are consistent
Similarity in potency across the group/category		H/M/L	Is there consistency in the potency and adverse effects in the data?	Data are consistent in terms of potency and adverse effects
Supporting data		H/M/L	Are supporting data provided e.g. mechanistic information, non-standard test data, data for other endpoints to demonstrate consistency	Supporting data demonstrate consistency and increase confidence in the read-across
Documentation of read-across			Is the documentation consistent, adequate and fit-for-purpose	Well documented read-across
Relationship between evidence and read-across hypothesis			Do all lines of evidence support the justification of similarity sufficient for a read-across argument to be accepted	Lines of evidence are provided and are consistent

APPENDIX D

The expanding role of read-across approach: selected case studies

Many case studies have been published for a range of endpoints over the last decade, including both scientific investigations and more regulatory-oriented or regulation-supporting studies. The examples provided below are drawn from historical assessments that informed the development of this guidance. They should not be viewed or used as models for applying the guidance; instead, future practice should follow the methodology described herein.

Blackburn et al. (2011) first published a series of case studies to demonstrate the analogue identification and evaluation approach in (Wu et al., 2010). Ehrlich (2015) applied docking simulations in addition to experimental data to evaluate zearalenone and its metabolites as a group with respect to its estrogenic potential. *'(*Brandt et al., 2016*) evaluated a WoE approach to assess the persistence characteristics of certain phenolic benzotriazoles. (*Gautier et al., 2020*) showcased use of a defined approach as part of a read-across for a resorcinol case study for the skin sensitisation endpoint. (*Gelbke et al., 2018*) evaluated a category of methacrylates developed to showcase an example assessment prepared to meet the needs of a REACH submission. (*Grimm et al., 2019*) used phenotypic and transcriptomic assay data to demonstrate bioactivity similarity in a set of glycol ethers'* (Tate et al., 2021). Atwood et al. (2019)) presented perspectives in evaluating cancer hazards for 13 haloacetic acid, water disinfection by-products. Pestana et al. (2021)) showcased how NAM data could reduce uncertainty in the read-across within a category of tetraconazoles to address the information requirements of a 90-day study outcome. Research Institute for Fragrance Materials (RIFM) safety assessments routinely apply read-across in their evaluations (Api et al., 2015).

Additional case studies are described in a number of articles notably by Schultz and Cronin (2017), Schultz, Richarz, and Cronin (2019) as well as a number of workshop articles (see (Ball et al., 2020; Chesnut et al., 2018; Mahony et al., 2020; Rovida, 2021)). Case studies to build acceptance in the use of NAM include many that have been published by the OECD under the auspices of their IATA Case studies programme (ref to the IATA work-space). One example includes Webster et al. (2019) which shows how lines of evidence across analogues and vertically for an analogue can be helpful to substantiate the category similarity and build confidence in the read-across prediction of oestrogenicity of hindered phenols. A summary of the programme and the learnings was described by Sakuratani et al. (2018). Several examples are presented as well in the EFSA guidance on the use of the weight of evidence approach in scientific assessments (EFSA Scientific Committee, 2017).

Table D.1 includes a selection of some of the relevant case studies, which are further described in detail (Sections **D.1–D.6**). The first three cases are directly related to EFSA activity (e.g. outsourced projects detailed below that specifically addressed the applicability and performance of read-across, by exploring different strategies using rich data from pesticide active substances and their metabolites.), while the subsequent three cases were developed within the OECD IATA project.

TABLE D.1 Examples of case studies relevant to the application of read-across in risk assessment.

Endpoint	Topic	Metrics	Reference
In vitro genotoxicity (see **D.1**)	Prediction of genotoxicity of pesticide metabolites	Qualitative	OC/EFSA/PRAS/2016/01 (Benigni et al., 2020)
Carcinogenicity (see **D.2**)	Carcinogenic activity/potency of N-Nitrosoamines	Quantitative	EFSA CONTAM Panel (2023)
Repeated-dose toxicity (see **D.3**)	Liver and developmental toxicity of pesticides	Qualitative	OC/EFSA/SCER/2021/04 (Irwan et al., 2024)
Neurotoxicity (see **D.4**)	Parkinsonian hazard liability of deguelin	Semi-quantitative	OECD (2020a)
Neurotoxicity (see **D.5**)	Neurotoxicity of Azoxystrobin	Qualitative	OECD (2020b)
Develop/Reproductive Toxicity (see **D.6**)	Developmental toxicity of methyl hexanoic acid	Qualitative	OECD (2020c)

D.1 | IN VITRO GENOTOXICITY OF PESTICIDE METABOLITES

Research on read-across as applied to genotoxicity was performed within a project funded by EFSA,[27] aimed at evaluating the use of in silico models for predicting the genotoxicity of pesticide active substances and their metabolites (Benigni et al., 2020). While a comprehensive toxicological dossier is developed for pesticides active substances, often none or only limited information about toxicological properties of their metabolites is available. Thus, EFSA has proposed the use of (Q)SARs and read-across for the assessment of genotoxic potential of all metabolites as a first step in the process for setting residue definition for risk assessment. In the investigation, two read-across approaches were proposed and evaluated for their predictive ability (for predicting in vitro Ames mutagenicity and in vitro chromosome aberrations):

[27]Evaluation of the applicability of existing (Q)SAR models for predicting the genotoxicity of pesticides and similarity analysis related with genotoxicity of pesticides for facilitating of grouping and read across (OC/EFSA/PRAS/2016/01).

1. In one approach, the similarity between a metabolite with genotoxicity data-gaps and the parent pesticide was assessed based on three physical chemical/structural parameters: molecular weight, logP, dice/atom centred structural similarity.
2. In another approach multiple sources of information related to a MoA hypothesis were combined and decision theory approach (based on Dempster–Shafer theory) was applied to obtain a WoE final outcome, together with an estimate of uncertainty.

Both read-across strategies predicted Ames mutagenicity with large success, whereas the performance with regard to predictions of in vitro chromosomal aberrations showed differences and overall were not satisfactory. The reason for the lower performance was likely due to low data quality and the limited size of the chromosomal aberration database, a discrepancy also recognised in the EFSA guidance on genotoxicity testing (EFSA Scientific Committee, 2011).

One read-across approach for predicting the genotoxicity of 'metabolites' (with data gaps), based on the information available for the parent pesticides, was designed ad hoc for the project. The key step was the assessment of the similarity between metabolites (with data gaps) and their parent substances. A metabolite was considered similar to the parent substance if they were simultaneously similar for three parameters (molecular weight, logP, dice/atom centred structural similarity) (within established cut-off values). When the three conditions were fulfilled, the genotoxicity of the metabolite was inferred from that of the parent substance. When not, a majority vote from a larger number of analogues was worked out. In a subsequent work (Benigni, 2019), the approach was validated on a large database of curated Ames mutagenicity results. For around 2,000 chemicals for which the similarity criterion was applicable, the predictivity of read-across was high (overall accuracy: 0.85). A second read across approach to estimating the genotoxicity of metabolites -starting from the data on their parent pesticides- was evaluated as well. Central to the second strategy was the systematic use of a decision theory approach (based on Dempster-Shafer theory (DST)) to estimate uncertainty and combine multiple sources of information to obtain a weight-of-evidence (WoE) outcome (Rathman et al., 2018). Specifically, in this strategy diverse evidence sources for parent and analogue substances are collected and the biological similarity between parent active substances and metabolites is assessed based on the relevant pesticidal MoA to verify that both target and analogue(s) operate within the same MoA. The MoA is coded with substructural motifs typical of the various classes of pesticides. Similarity measures based on chemical structure, molecular/physicochemical properties, and metabolic reactivity are also determined. Metabolic similarity is calculated based on the proportion of potential metabolic reaction sites shared by two chemicals.

Overall, both read-across strategies were able to predict Ames mutagenicity, whereas the quality of predictions for in vitro chromosomal aberrations was not as satisfactory. It was hypothesised that the main reason for this difference was the lower quality and more limited size of the chromosomal aberrations database.

D.2 | CARCINOGENICITY OF N-NITROSAMINES

Recently, read-across and related trend analysis has been used by EFSA to fill data gaps in the assessment of the carcinogenic risk of N-Nitrosamines (N-NA) present in food (EFSA CONTAM Panel, 2023). Most N-NAs undergo a Cytochrome P450 (CYP)-mediated oxidation which is a key event in bioactivation. Usually, N-NAs are metabolised via α-hydroxylation and give rise to diazonium ions that can produce DNA adducts and ultimately generate mutations and initiation of carcinogenesis. Modulating factors that influence the reactivity are known (e.g. presence of acidic groups, bulky/unmetabolisable substituents at the α-carbon, branching or bulky substituents in the vicinity of the α-carbon). Identification and evaluation of analogues was supervised applying the above mechanistic/structural knowledge. In several cases available mutagenicity, metabolic and DNA adducts data contributed to the evaluation of analogues. The calculation of Dice similarity contributed to the identification/confirmation of the source substances as well. On this basis, read across and trend analysis permitted the prediction of the carcinogenic activity/potency (TD50) of 18 N-NAs without data.

D.3 | REPEATED DOSE TOXICITY (LIVER AND DEVELOPMENTAL TOXICITY) OF PESTICIDES

Within this project,[28] a read-across assessment framework was developed that modularly integrates existing information on chemical and mechanistic properties as well as observed metabolites (Irwan et al., 2024). A data-rich reference class of pesticides was used to evaluate the performance of the read-across workflow. As such, a modular read-across approach for identifying source substances was developed and applied related to repeat-dose toxicity (liver toxicity, developmental toxicity and general unspecific systemic toxicity e.g. as indicated by changes in body weight), as well the added values of evaluating in vivo ADME data. The modules reflected chemical properties, mechanistic properties and metabolites. In this manner, the following modules were combined and evaluated in different case studies:

1. Chemical similarity
2. Substructure search
3. Mechanistic evidence, both qualitative similarity and similarity in terms of potency
4. Metabolic pathway, e.g. shared or similar metabolites

[28]Identification of the applicability domain (in terms of toxicological endpoints and chemical space) for the use of read-across in food safety (OC/EFSA/SCER/2021/04).

Three different combinations were tried out (see **Figure D.1** adapted from (Irwan et al., 2024)):

- Overall it was learned that the combination of chemical and mechanistic similarity identified the majority of relevant source substances and was particularly successful when the MoA of the target substance was known (**Case study 1a** and **1b**). The demonstration of mechanistic similarity is highly dependent on the completeness and availability of data, and low data density could be used as a measure of uncertainty.
- Starting the identification of source substances from NAM data (e.g. ToxCast outputs) was excessively non-specific in the case, resulting in an extremely large and unmanageable number of candidate source substances (**Case study 2**).
- Integration of common metabolites was a very efficient way to reduce the number of source substances to the most relevant substances. However, it was found that a loss of relevant source substances could occur mainly due to data gaps (**Case study 3**).

The assessment of general unspecific systemic toxicity using chemical and biological similarity was difficult, however vast NAM data confirmed an overall low activity.

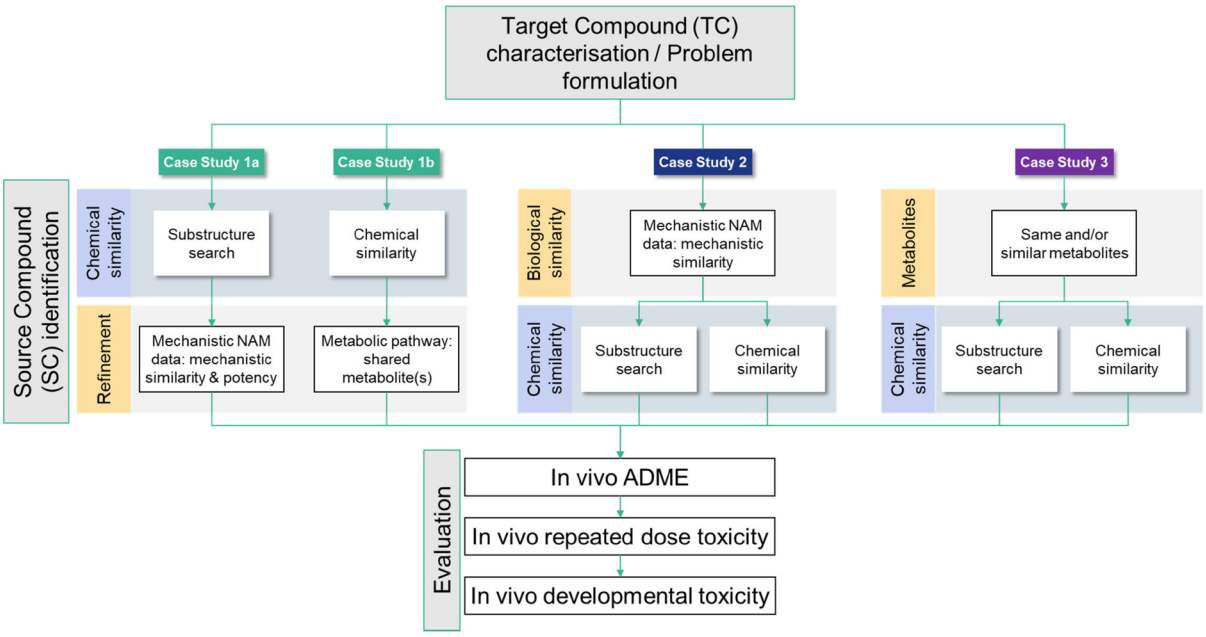

FIGURE D.1 Workflows applied within a modular read-across approach (adapted from (Irwan et al., 2024)).

The combination of chemical and mechanistic similarity identified the majority of relevant source substances and was particularly successful when the MoA of the target substance was known. Mechanistic similarity is highly dependent on the completeness and availability of data, and low data density could be used as a measure of uncertainty. The identification of source substances from biological NAM data (e.g. ToxCast) was too unspecific in the case studies presented in this project, resulting in an overwhelming number of candidate source substances. The assessment of low toxicity substances using both approaches was also challenging, but the NAM data confirmed an overall low activity. The integration of common metabolites was a very efficient way to reduce the number of source substances to the most relevant substances. A disadvantage of this approach is the loss of interesting source substances mainly due to data gaps. Overall, the developed workflows are recommended for the identification of source substances and are complementary to the workflows proposed e.g. by EU-ToxRisk (Escher et al., 2019). An integration of alternative approaches to assess differences in bioavailability is recommended, e.g. by using physiologically based kinetic modelling approaches.

D.4 | PARKINSONIAN HAZARD LIABILITY OF DEGUELIN

Deguelin can induce parkinsonian-like phenotypes in rats. Whether deguelin has such a parkinsonian hazard manifestation in humans is currently unclear and therefore deguelin was the target substance for this IATA case study (OECD, 2020a).

Epidemiological studies indicate that exposure of workers to rotenone is statistically associated with increased incidence of Parkinson disease; moreover, rotenone is used to induce Parkinson phenotypes in experimental animals. Therefore, rotenone was used as the source substance for the read-across study.

An AOP based on data of rotenone as a stressor is known/accepted, according to which inhibition of the mitochondrial complex I of nigrostriatal neurons leads to parkinsonian motor deficits.

In this read-across analysis, the scientific hypothesis was that deguelin would give similar biological interactions and activation of KEs as rotenone in the selected test battery that represents the KEs of the AOP, but with differences in potency. The testing strategy contains in silico (for MIE binding site and PBK models) and in vitro assays (key events).

Based on the AOP, structural modelling approaches were used to define the binding of rotenone and deguelin to mitochondrial complex I, the molecular initiating event of the AOP. Previously established and routinely applied assays that reflect the various key events in this AOP were defined, including multiple human-based in vitro test systems to monitor the effects on mitochondrial effects of rotenone and deguelin. Moreover, high content imaging-based approaches to measure the degeneration of neuronal neurites were applied. Finally, both toxicokinetic evaluation of cellular exposure to rotenone and deguelin as well as PBK modelling has been used to evaluate the relevance of the observed effects in vitro towards a likely in vivo exposure situation.

Results from in silico docking to mitochondrial complex I indicate that rotenone and deguelin are highly similar structurally and share a common pharmacophore for the binding. Rotenone and deguelin have similar in vivo metabolism profiles and toxicokinetic behaviour in vitro and in vivo. Both substances inhibit complex I activity and cause mitochondrial dysfunction, with rotenone being ~3 times more potent than deguelin. Rotenone is also more potent than deguelin in disrupting neurites. It was concluded that deguelin has a similar mode-of-action as rotenone but is less potent in its action.

D.5 | POTENTIAL NEUROTOXICITY OF AZOXYSTROBIN AND OTHER STROBILURINS

The synthetic strobilurin fungicides are derived from the naturally occurring strobilurin A and B. The strobilurins bind to the quinol oxidation site of cytochrome b of complex III (CIII) of the mitochondria which is also their fungicidal MoA. There are some signals of potential neurotoxicity from in vitro studies by a CIII-mediated mechanism.

The objective of this study is, by means of NAM data, to characterise the potential CIII-mediated neurotoxicity of azoxystrobin by read-across (OECD, 2020b).

The source substances are other strobilurin fungicides (pyraclostrobin, picoxystrobin, trifloxystrobin and kresoxim-methyl). The substances in the category share similar chemical structure, similar pesticidal MoA, similar toxicophore, similar neurotoxic potential and similar toxicokinetics to azoxystrobin. Furthermore, in vitro testing was conducted on Antimycin A, a well-established CIII inhibitor with neurotoxic effects, which serves as a reference substance for this MoA. The degree of in vivo inhibition of the mitochondrial respiratory system depends on the respiratory activity and thus the tissues like brain can be more susceptible if exposed.

Existing regulatory in vivo data was collected for the source and target substances with a focus on ADME, neurotoxicity as well as target organ toxicity data. The source substances do neither show signs of neurotoxicity in neurotoxicity studies nor in other repeat dose toxicity studies.

The scientific hypothesis of this case study is: Can the absence of a neurotoxic potential (as detected with a TG424 study) mediated by inhibition of Complex III of the mitochondria be predicted by toxicodynamic and toxicokinetic NAM data? The hypothesis is supported by mechanistic data, anchored to a putative AOP (based on the OECD adopted AOP on CI inhibition leading to parkinsonian disorder), and kinetic PBK data.

Inhibition of CIII complexes measured by oxygen consumption, by the target substance azoxystrobin seemed to be slightly less strong than by the source substances pyraclostrobin and picoxystrobin, while antimycin A resulted in a much stronger inhibition. This was confirmed with whole cells as well. Effects on membrane potential were marked by Antimycin A and orders of magnitude less with the target and source substance. Effects on glycolysis and cell viability were similar between the substances. The target substance was negative in the neurite outgrowth assay using SH-SY5Y cells, while some of the source substances showed weak effects. However, neither the target nor the source substances were considered neurotoxic in the neuritetox assay conducted in LUHMES cells.

The kinetic data and simulations confirm comparable toxicokinetics and that the exposure of the brain to the strobilurins is limited, resulting in maximally twice the plasma concentration.

Overall, based on the generated data on toxicokinetics and toxicodynamic data, there is no evidence for a stronger neurotoxic potential of azoxystrobin mediated by a complex III inhibitory mode of action as compared to the source compounds. Since the source compounds do not show neurotoxicity in vivo, it is concluded that also the target compound azoxystrobin is not a neurotoxicant.

D.6 | DEVELOPMENTAL TOXICITY OF METHYL HEXANOIC ACID

2-Methylhexanoic acid (MHA) is a substance for which developmental and reproductive toxicity test (DART) data are lacking. Structural analogues that have this data were retrieved in order to explore the possibility to read across information of these source substances to MHA (OECD, 2020c).

The following structural related aliphatic carboxylic acids with in vivo developmental and/or reproductive toxicity data were retrieved: 2-ethylhexanoic acid (EHA), 2-propylpentanoic acid (VPA), 2-propylheptanoic acid (PHA), 2-ethylbutanoic acid (EBA), 4-pentenoic acid (PA), 2-propyl-4-pentenoic acid (4-ene-VPA), and 2-dimethylpentanoic acid (DMPA). Some of these analogues proved to be clear developmental toxicants, i.e. VPA, PHA, EHA, and 4-ene-VPA, while others were identified as not being toxic to development, i.e. EBA, PA, and DMPA; i.e. they did or did not induce neural tube defects upon in vivo exposure. Since structural similarity alone does not correspond one-to-one to a similarity of developmental toxicity, MHA and all the selected source substances were tested in a battery of in vitro tests with clear relevance to developmental toxicity, i.e. the zebrafish embryo test (ZET), mouse embryonic stem cell test (mEST), iPSC-based neurodevelopmental model (UKN1) and a series of CALUX reporter assays, that were combined with toxicokinetic models to calculate effective cellular concentrations and associated in vivo exposure doses.

The potential to inhibit histone deacetylase in ZET, mEST and UKN1 models was also investigated, as this enzyme is postulated to be the molecular initiating target leading to neural tube defects observed with these analogues.

The NAM results show that VPA, PHA, EHA, and 4-ene-VPA were correctly predicted as in vivo developmental toxicants, and EBA, and DMPA as non-developmental toxicants. Based on NAM-based similarity, it was concluded that MHA may not be fully negative for developmental toxicity.

D.7 | LESSONS LEARNED FROM CASE STUDIES

The case studies presented more in detail above employ a variety of approaches to Steps 3 and 4 of read-across (identification and evaluation of analogues). Analyses relative to in vitro genotoxicity of pesticide metabolites and (genotoxic) carcinogenicity of N-nitrosamines use mainly cheminformatics approaches, exploiting the fact that the underlying mechanisms of action are known in their general lines. The three IATA case studies are relative to more peculiar mechanisms of action, so they make specific reference to AOP communality between target and source substances. This communality is validated/supported by different NAMs (e.g. in vitro assays, in silico PBK or docking approaches, etc.). Moreover, the IATA case studies provide a standardised table with uncertainties for composing elements and overall. In the procurement study related to repeated dose toxicity (liver and developmental toxicity) of pesticides, various combinations of chemical similarity, biological NAM and physiologically based kinetic modelling approaches were investigated, and results were presented.

In conclusion, the selection of case studies shows how read across can be tailored in different ways based on the specific scientific issue to be solved. The need for a systematic analysis of uncertainty is emphasised.

APPENDIX E

Glossary of terminologies and definitions

Adverse Outcome Pathway (AOP)**	An AOP describes a sequence of events commencing with initial interactions of a stressor with a biomolecule within an organism that causes a perturbation in its biology.
Analogue (or analogue substance)**	Is one substance (chemical) that has been identified as exhibiting similarity to another substance.
Analogue approach	The term analogue approach is used when read-across is employed between a few, very structurally similar substances for which it is not possible to establish a trend or a regular pattern. As a result of the structural similarity, a given (toxicological or other) property of one substance (the source) is used to predict the same property for another substance (the target), for which information on this property is not available but is needed to fulfil the information requirement. The outcome of a study conducted with the source substance is read-across for all investigated parameters to the target substance. A worst-case approach may also be used.
Applicability domain	The set of inclusion/exclusion rules that identify the ranges of values within which a reliable prediction can be made for category members.
Bias	Three types of bias are addressed: 1. Analogue substance selection. Information from (an)other suitable analogue substance(s) which is significantly different for relevant property(ies), and thereby reduce confidence in the proposed prediction. 2. Study selection. Information from other studies than the one proposed to be used as source study, which give rise to a higher concern. 3. Independent variable. The results of the measurement or estimation for the independent variable used to describe a regular pattern are systematically and inappropriately altered in category members with certain structural features. This may have an influence on the prediction.
(Bio)transformation	A series of chemical changes in a substance as a result of enzymatic or other activity in a living organism. The term 'transformation' used for environmental endpoints refers to abiotic and biotic degradation.
Category approach	Used when read-across is employed between several substances that have structural similarity. These substances are grouped together on the basis of defined structural similarity and differences between the substances. As a result of the structural similarity, one or more toxicological properties are proposed to be similar or to follow a regular pattern. The predictions are made within the group for the target substance(s) based on the observed regular pattern. Alternatively, the prediction is based on a read-across from a category member in a conservative manner (worst case).
Category justification	Reasoning and associated supporting evidence that are provided to verify the scientific validity and robustness of the category hypothesis.
Chemical space	In the context of this guidance refers to the chemicals, the properties and characteristics of the substances considered in the read-across.
Constituent	A discrete chemical structure that can be clearly distinguished from its stereoisomers, regioisomers, and constitutional isomers.
Data gap	In the context of this guidance, 'data gap' refers to the data to be filled by read-across as defined in the problem formulation. In contrast, missing (supporting) information or data refers to the properties of the target and/or source substances to support read-across.
Data matrix	A table that summarises all available study results of the source and target substances requirement/endpoint and including planned studies. The data should be arranged to reflect the regular pattern identified and used in the prediction. The IUCLID dossier should contain (robust) study summaries of each study referred to in the data matrix to allow an independent assessment of the data.
Endpoint**	Any physicochemical, biological, or environmental property that can be measured/modelled. An endpoint could be determined by different experimental protocols and under different experimental conditions.
Grouping' or 'Chemical grouping'**	The process of identifying a collection of substances that are likely to be similar or follow a regular pattern as a result of similarity (see definition).
Interpolation**	The estimation of a value for a member using measured values from other members on 'both sides' of that member within the defined category spectrum.
Mechanism of Action**	A detailed molecular description of the mechanistic interaction through which a substance/molecule produces its effect.
Mode of Action**	A biologically plausible sequence of key events at different levels of biological organisation, starting with the exposure to a chemical and leading to an observed (adverse) effect.
Negative read-across / Prediction of absence of effect(s)	This term refers to the situation where no effects have been observed in a source study and this result, i.e. absence of effect(s), is read-across to a target substance.

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New Approach Methodologies	Refers to any in chemico, in silico or in vitro method, (including high throughput screening tools and high-content methods such as 'omics technology), used as stand-alone testing methods or in combination (e.g. incorporated in Integrated Approaches to Testing and Assessment (IATA), Defined Approaches (DA) or within Next generation Risk Assessment framework (NGRA)), for a specific biological/toxicological endpoint to provide information on hazard, exposure or risk assessment, and contributing to the 3Rs principles on replacement, reduction and refinement of animal testing (*adapted from* (Westmoreland et al., 2022; ECHA, 2023b; SCCS 2023b)).
Omics**	In the context of this guidance document, 'omics refers to technologies that are used to measure a broad range of molecular responses to chemical exposure. Widely used approaches include transcriptomics (study of expression of multiple genes) and metabolomics (study of levels of multiple endogenous metabolites and the biochemical processes in which they are involved in), within a cell, tissue or organism.
Order (within the category)	To predict a property within a category of substances, an order has to be established among the category members. As structural similarity is the basis for read-across this order has to be based on a variable directly linked to the allowed structural differences in the group (e.g. the number of carbon atoms in a side chain or a suitable physical chemical property).
Property	In the context of this document, property is considered to refer to inherent characteristics of the substance, which can be studied in a defined experimental study type. These characteristics may relate to physicochemical, environmental fate or (eco)toxicological aspects. The properties of a substance can be determined from the results of experimental studies.
Quantitative Structure Activity Relationship (QSAR)**	A QSAR is a mathematical model (often a statistical correlation) relating one or more qualitative and/or quantitative parameters derived from chemical structure to a qualitative or quantitative measure of. QSARs are quantitative models yielding a continuous or categorical result.
Similar substance (or chemical)	In the context of this guidance refers to similarity in regard to structure and/or functionality against the endpoint of interest
Similarity**	Several factors should be considered when evaluating similarity. These factors can include, structure, physico-chemical properties, chemical reactivity profile, bioactivity, conventional toxicological profile (including metabolism) and ADME/TK.
Source substance (used also as source chemical, compound or analogue**)	A substance that has been identified as appropriate for use in a read-across based on similarity to the target substance and existence of relevant data.
Structural alert**	Term used in the context of the structure-activity relationships, referring to the substructure associated with the presence of a biological activity.
Target substance (used also as target chemical or compound)	Substance of interest for which data gaps exist that need to be addressed.
Transformation	A series of chemical changes in a substance as a result of biotic or abiotic degradation.
Uncertainty**	According to EFSA Guidance on Uncertainty Analysis in Scientific Assessments (EFSA Scientific Committee, 2018a) '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'.
Weight of Evidence (WoE)	A stepwise process/approach of collecting and weighing evidence to reach a conclusion on a particular problem formulation including assessment of the degree of confidence (OECD, 2019).

**OECD Guidance on Grouping of Chemicals, Third Edition, under development (Version for consultation, March 2024).

The **references** used for the definition of terms, unless specified in the table, include: (ECHA, 2017c; ECHA, 2022a).