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SCIENTIFIC OPINION

Scientific Opinion on the application of physiologically based kinetic (PBK) modelling for the quantitative in vitro to in vivo extrapolation (QIVIVE) of developmental neurotoxicity in vitro battery (DNT IVB) data for pesticide active substances

EFSA Panel on Plant Protection Products and their Residues (PPR) | Tamara Coja | Pauline Adriaanse | Judy Choi | Antonio Finizio | Maeva Giraudo | Thomas Kuhl | Emily McVey | Francesca Metruccio | Martin Paparella | Silvia Pieper | Eugenio Scanziani | Ivana Teodorovic | Paul Van der Brink | Martin Wilks | Keyvin Darney | Jean-Lou C.M. Dorne | Antonio Hernandez-Jerez | Nynke Kramer | Alicia Paini | Emanuela Testai | Jochem Louisse

Correspondence: Ask a Question

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

Abstract

EFSA requested the Panel on Plant Protection Products and their Residues (PPR Panel) to produce a Scientific Opinion on the application of physiologically based kinetic (PBK) modelling for the quantitative in vitro to in vivo extrapolation (QIVIVE) of data from the 17-assay developmental neurotoxicity in vitro battery (DNT IVB) for pesticide active substances. PBK modelling-supported QIVIVE is essential for the integration of in vitro data in hazard and risk assessment and may be conducted via forward dosimetry (estimating internal exposure from external exposure to a chemical) or reverse dosimetry (deriving an external exposure from an internal exposure). The request was accomplished via targeted expert discussions and EFSA-internal and -external review. A scientifically robust QIVIVE requires accurate characterisation of two interrelated exposure metrics: the in vitro concentration eliciting a biological response (e.g. free or cellular concentration) and the corresponding in vivo internal concentration predicted by PBK modelling that reflects the same biologically relevant exposure. These metrics are influenced by chemical-specific properties, assay design and physiological variability, requiring a case-by-case assessment of uncertainties. Each QIVIVE assessment should include, as a minimum, a low-tier PBK model using conservative assumptions to avoid underestimation of internal exposure. Where sufficient kinetic data exist, higher tier models may be applied to enhance prediction accuracy. All modelling parameters, assumptions and validation steps must be transparently documented to facilitate regulatory appraisal. QIVIVE outcomes are to be documented in the overall weight of evidence for the DNT assessment. The PPR Panel identified the following three key uncertainty domains: (a) defining the appropriate in vitro exposure metric, (b) predicting internal exposure metrics via PBK modelling and (c) aligning PBK modelling-derived internal exposure with the in vitro exposure. Addressing these uncertainties will strengthen regulatory confidence in using DNT IVB data for hazard and risk assessment of pesticide active substances.

KEYWORDS

developmental neurotoxicity, DNT IVB, IATA, NAMs, PBK modelling, pesticide active substances, QIVIVE

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SUMMARY

The developmental neurotoxicity in vitro battery (DNT IVB) consists of in vitro test systems based on human and rat cells with 17 readouts and these allow an assessment of the potential disturbance of key neurodevelopmental processes resulting from chemical exposure (OECD, 2023a). The OECD published in 2023 their *Initial Recommendations on Evaluation of Data from the DNT IVB*, aiming to provide guidance on the evaluation and interpretation of results from the DNT IVB for the identification of substances with DNT potential.

To allow a quantitative interpretation of the DNT IVB data and its integration in the chemical hazard and risk assessment process, the application of physiologically based kinetic (PBK) modelling is essential, allowing a so-called quantitative in vitro to in vivo extrapolation (QIVIVE), i.e. a translation of in vitro exposure information into equivalent in vivo external (e.g. oral) exposure. Currently, examples of QIVIVE have been described in the scientific literature, but QIVIVE is not generally applied in regulatory hazard and risk assessment. With regards to QIVIVE, no harmonised approach or guidance is available. In 2025, the OECD published a document titled *Principles of quantitative in vitro to in vivo extrapolation (QIVIVE) – As example applied to the DNT IVB* (OECD, 2025), providing an overview of QIVIVE principles to facilitate the incorporation of DNT IVB data into chemical assessment. However, in that document, no specific guidance is provided for performing QIVIVE of DNT IVB data for hazard and risk characterisation, and for such a purpose, specific criteria are required to be developed by scientific advisory bodies and regulatory authorities while applying relevant regulatory frameworks.

The EFSA Panel on Plant Protection Products and their Residues (PPR Panel) was self-tasked to develop a Scientific Opinion on the application of PBK modelling for the QIVIVE of DNT IVB data for the hazard and risk assessment of pesticide active substances in Europe, taking into consideration available guidance documents on PBK modelling and the ongoing activities at OECD level in relation to the DNT IVB. According to the Terms of Reference, the Scientific Opinion should describe minimum data requirements together with preferred fit for purpose approaches, best practices to determine the relevant in vitro exposure parameters and uncertainties to perform QIVIVE of DNT IVB data, allowing their integration in pesticide human health hazard and risk characterisation.

To address this request, a PPR Panel working group (WG) was established supported by experts in QIVIVE, toxicokinetics, PBK modelling, chemical distribution in in vitro test systems, mammalian toxicology and hazard and risk assessment of pesticide active substances. As only a few DNT QIVIVE case studies are available from the scientific literature and QIVIVE has not been routinely applied in regulatory hazard and risk assessment, an expert knowledge-based approach was used for this self-tasked mandate. The draft Scientific Opinion developed by the PPR Panel WG underwent public consultation and was updated accordingly to produce this document.

The main aspects for QIVIVE include the selection of an in vitro exposure metric[1] and an internal exposure metric that are to be connected in the QIVIVE. In order to link an in vitro exposure metric to an in vivo exposure metric, one needs to assess (1) the kinetic basis for QIVIVE, either using the maximum (C_{max}) or steady-state (C_{ss}) concentrations or the area under the concentration-time curve (AUC; the product of concentration and exposure time), (2) the in vitro exposure metric for QIVIVE (medium or cell-associated concentration) and (3) the in vivo internal exposure (plasma and tissue/organ). The Panel recommends focussing QIVIVE on C_{max} or C_{ss} in plasma. However, it is helpful to also consider QIVIVE based on other exposure metrics (e.g. AUC) and compare related QIVIVE outcomes when analysing uncertainties, acknowledging that information to derive an in vitro AUC is most often not available. The PPR Panel also recommends linking the maximum in vitro unbound concentration in the exposure medium of the DNT IVB assay to the in vivo C_{max} or C_{ss} unbound plasma concentration predicted through PBK models. QIVIVE may also be performed through linking the maximum in vitro cell-associated concentration to the in vivo C_{max} or C_{ss} in tissue. If no information is available on such in vitro maximum unbound and cell-associated concentration, the nominal concentration may be applied provided that the uncertainties associated with the use of such an exposure metric are thoroughly assessed. It is noted that for an accurate QIVIVE, insights regarding in vitro distribution kinetics are essential, i.e. understanding fate of the test item in the in vitro test system.

With regards to PBK modelling, rat and human models are to be applied, and the modelling approach may be tailored to the relevant life stage, provided that sufficient data are available to allow such life-stage specific modelling. As a minimum, QIVIVE using a full body compartmental model (in line with a Tier 1 model as described in OECD, 2025) is recommended and may be sufficient for the case. Such a low-tier model should use conservative assumptions to avoid underestimation of internal exposure. If sufficient data are available, higher tier PBK models are recommended to be developed as well, provided that these do not result in larger uncertainties associated with the PBK modelling predictions.

Proposed minimum data requirements regarding chemical-specific PBK model parameters include fraction absorbed, intestinal uptake rate, fraction unbound in plasma, tissue:plasma partition coefficients and hepatic biotransformation rates (i.e. intrinsic clearance or K_m and V_{max})). These minimum parameters may not be necessarily sufficient to apply adequate modelling for all pesticide active substances since this aspect depends upon the chemical's specific absorption, distribution, metabolism, excretion (ADME) characteristics. For PBK model development and evaluation, this Scientific Opinion refers to the available OECD guidance documents. With regards to quality criteria for in vitro kinetic studies, key aspects include analytical methods (including appropriate sample preparation (extraction) and assessment of matrix effects, and application of validated analytical methods), test system characterisation, inclusion of reference/control chemicals and

[1]The Scientific Opinion uses the terms external exposure metrics, internal exposure metrics and in vitro exposure metrics. In the context of this Scientific Opinion external exposure metrics refer to external (oral) exposures, e.g. a toxicological reference value or a no observed adverse effect level, typically expressed in mg/kg bodyweight (per day). Internal exposure metrics refer to internal in vivo exposures, such as a concentration in plasma or in an organ or tissue. In vitro exposure metrics refer to exposure metrics in the in vitro test systems (of the DNT IVB), such as an unbound concentration in the medium or a cellular (cell-associated) concentration.

chemical-specific optimisation of experimental conditions. In addition, adherence to OECD GIVIMP (OECD, 2018) is critical. With regards to in silico approaches, adherence to OECD QSAR principles and the associated QSAR reporting framework is essential.

For each QIVIVE case, it is recommended to structure the information regarding uncertainties related to main building blocks of the QIVIVE, i.e. (1) the in vitro-based exposure metric, (2) the PBK modelling-predicted internal exposure metric and (3) linking the PBK modelling-based internal exposure metric to the in vitro exposure metric. Specific attention should be given to possible contribution of animal/human metabolites to DNT. Uncertainties related to the QIVIVE are to be transparently reported and their impact on the QIVIVE outcome is to be discussed. The QIVIVE outcome would then contribute to the overall body of evidence considered in the hazard and risk assessment of the pesticide active substance.

The Scientific Opinion proposes a framework to perform and report the QIVIVE and a related reporting template is available as an Annex to this Scientific Opinion to facilitate regulatory appraisal.

This Scientific Opinion concludes on recommendations for future steps, allowing to better define the DNT characteristics and the application of PBK modelling and QIVIVE while reducing uncertainties.

1 | INTRODUCTION

1.1 | Background and Terms of Reference as provided by the requestor

In June 2023, the OECD published the *Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity In Vitro Battery (DNT IVB)*, which is the result of a major endeavour from EFSA (PREV Unit), the US EPA and academic groups over the last years to standardise a battery of 17 in vitro assays for DNT assessment. The purpose of the OECD document is to provide guidance on the evaluation and interpretation of results from the DNT IVB for the identification of substances with DNT potential. However, no guidance is provided on the use of such data for hazard characterisation and risk assessment. For such purpose, specific criteria need to be developed by regulatory authorities based on the relevant regulatory frameworks.

In addition, the OECD document contains a series of Integrated Approaches to Testing and Assessment (IATA) case studies using the DNT IVB data for answering different regulatory problem formulations as a proof of concept. EFSA has supported chemical testing in the development of the current DNT IVB (Masjosthusmann et al., 2020) and developed IATA case studies for pesticides risk assessment and related recommendations (Crofton & Mundy, 2021; OECD, 2022a, 2022b).

In 2022, EFSA engaged relevant European stakeholders (170 representatives of EU Member States, ECHA, European Commission, NGOs and academia) in a workshop to define the next steps for using new approach methodologies (NAMs) in risk assessment with the ultimate goal to assess any regulated chemical for DNT using IATA and minimising the need for in vivo DNT studies.[2] Nevertheless, it was acknowledged that only few pesticides active substances were assessed through this framework (i.e. AOP-informed IATA) and that priority should be given to produce further data and develop IATA case studies with a risk characterisation problem formulation. Along these lines, in April 2023, EFSA launched a call for proposals[3] to obtain in vitro data on pesticide active substances for the DNT IVB and IATA case studies.

To allow a quantitative interpretation of the DNT IVB data and its integration in the chemical hazard and risk assessment process, the application of physiologically based kinetic (PBK) modelling is considered essential, allowing a so-called quantitative in vitro to in vivo extrapolation (QIVIVE), i.e. a translation of in vitro bioactive concentrations into equivalent external (e.g. oral) exposure levels. Currently, examples of QIVIVE have been described in the scientific literature, but QIVIVE is generally not applied in regulatory hazard and risk assessment. So far, no harmonised approach or guidance is available on how to perform QIVIVE.

In order to perform PBK modelling-facilitated QIVIVE of DNT IVB data for a given pesticide active substance, a reliable PBK model for that active substance is needed. PBK models require data on system-dependent parameters (such as physiological parameters) as well as on chemical-specific parameters related to the kinetic processes, i.e. the absorption, distribution, metabolism and excretion (ADME). Such chemical-specific parameter values can be obtained based on in vivo and in vitro studies and on in silico predictions. Various guidance/guideline documents related to PBK modelling (EMA, 2018; FDA, 2018; OECD, 2021; WHO, 2010) are available, which mainly focus on the characterisation, application and reporting of the PBK models and the reporting of their simulations. These documents generally do not provide guidance on the studies required to obtain reliable chemical-specific PBK model parameter values.

Requirements related to the chemical-specific kinetic data may, among others, depend on the regulatory application and the required level of certainty in the PBK model simulations.

For pesticide active substances, the availability of in vivo kinetic data for PBK model development and model evaluation is rather limited, indicating that PBK model development for QIVIVE of DNT IVB data of pesticide active substances is expected to be based to a large extent on data from in vitro studies and in silico predictions. However, no guidance is available on how such studies should be performed to provide reliable PBK model input data.

The choice of the in vitro dose metric (nominal concentration, unbound concentration, cellular concentration) impacts the outcome of the QIVIVE. Different approaches have been described in the scientific literature, but no harmonised approach is available to be applied in a regulatory setting. In vitro biokinetic studies can help to better characterise the in vitro exposure and select the most appropriate in vitro dose metric for the QIVIVE.

This self-mandate aims to provide a Scientific Opinion of the PPR Panel on the use of PBK modelling for the QIVIVE of DNT IVB data for human health risk assessment of pesticide active substances in Europe. It aims to describe the preferred approach for the QIVIVE as well as a description of the minimal requirements related to the chemical-specific input data for the applied PBK models. It also aims to provide guidance to generate adequate kinetic data and develop modelling approaches to allow for QIVIVE of DNT IVB data. This will allow preparedness for assessing the data and its future integration in pesticides hazard and risk assessment.

Terms of Reference

In accordance with Article 29(1) of Regulation (EC) No 178/2002, the European Food Safety Authority requests its Scientific Panel on Plant Protection Products and their Residues (PPR) to provide a Scientific Opinion on the application of physiologically based kinetic (PBK) modelling for the quantitative in vitro to in vivo extrapolation (QIVIVE) of developmental

[2] https://www.efsa.europa.eu/en/events/european-stakeholders-workshop-new-approach-methodologies-nams-developmental-neurotoxicity.
[3] https://etendering.ted.europa.eu/cft/cft-display.html?cftId=13967.

neurotoxicity in vitro battery (DNT IVB) data for the hazard and risk assessment of pesticide active substances in Europe, taking into consideration available guidance documents on PBK modelling and the ongoing activities at OECD level related to the DNT IVB.

The Scientific Opinion should describe the data required, the preferred fit for purpose approach, best practices and uncertainties to perform the QIVIVE of DNT IVB data for integration in pesticide human health risk assessment, including definition of:

1. Minimal data requirements for chemical-specific input parameter values for a fit for purpose PBK model applied for the QIVIVE and quality criteria for kinetic studies to obtain them.
2. The needed in vitro biokinetic studies to determine the relevant in vitro exposure parameters for the QIVIVE of the assays included in the DNT IVB.
3. A preferred approach to perform and report the QIVIVE, including (a) selection of the PBK model and parameter values, (b) evaluation of the PBK model simulations, (c) selection of in the vitro dose metrics for translation, and (d) evaluation of the QIVIVE outcomes.
4. Main potential sources of uncertainties of the proposed QIVIVE for pesticide human health risk assessment, including those related to the possible derivation of reference points.

1.2 | Interpretation of the Terms of Reference

An EFSA PPR Panel WG was established to address the Terms of Reference (ToRs) as described in Section 1.1. The ToRs were discussed by the WG, interpreted and agreed upon as described below.

The Scientific Opinion will focus on the regulatory context of pesticide active substances at the EU level. It will address the application of PBK modelling for QIVIVE by translating in vitro concentrations into equivalent external (oral) exposure levels. This approach aims to enhance the quantitative interpretation of pesticide-induced effects in the DNT IVB assays and therefore it is distinct from its broader scientific use in the extrapolation of in vitro toxicity data. The focus will be on chemical-based pesticide active substances, whereas the other pesticide types (e.g. plant extracts, nanoparticles or microbial-based substances) remain outside its scope.

The Scientific Opinion does not address the interpretation of adversity regarding the effects measured in the DNT IVB. In other words, toxicokinetic aspects of QIVIVE are addressed but not toxicodynamic aspects.

ToR 1: Minimal data requirements for PBK model parameterisation

- The Scientific Opinion will describe the minimal data requirements for chemical-specific input parameters in PBK models used for QIVIVE of DNT IVB data. It will provide insight into the necessity of in vitro, in silico and in vivo kinetic data[4] for PBK model parameterisation while recognising that a universal minimal dataset may not always be sufficient for every pesticide active substance. Chemical-specific considerations and model complexity (lower tier vs. higher tier) should be assessed on a case-by-case basis.
- The Scientific Opinion will provide insight into the quality criteria for in vitro kinetic studies used in PBK model parameterisation, recognising the absence of specific guidance in this area. Key aspects include test system quality control, optimisation of kinetic studies design, assessment of chemical behaviour and in vitro distribution kinetics, and analytical considerations. However, the Scientific Opinion will not cover the quality assessment of the DNT IVB studies.
- The OECD's *Initial Recommendations on Evaluation of Data from the DNT IVB* (OECD, 2023a) provides recommendations on determining nominal effect concentrations in DNT IVB assays, which may serve as a starting point for QIVIVE although QIVIVE can be applied to any tested concentration.
- QIVIVE will focus exclusively on oral exposure as this route allows direct comparison with no observed-adverse effect levels (NOAELs), lowest observed adverse effect levels (LOAELs), benchmark dose lower confidence limits (BMDLs) and related toxicological reference values (TRVs)[5] derived from animal studies using oral exposure, the major exposure route applied in toxicity studies for pesticide active substances. Also, in vivo ADME studies for pesticide active substances are mainly performed for the oral exposure route. Other exposure routes are thus outside the scope of this Scientific Opinion.

ToR 2: In vitro biokinetic studies for QIVIVE of DNT IVB assays

- In this Scientific Opinion, in vitro biokinetic studies are defined as studies to assess the distribution of a test item within an in vitro test system of the DNT IVB. In this regard, in vitro biokinetic studies (i.e. in vitro distribution studies) are different from in vitro ADME studies that aim to provide chemical-specific kinetic data for PBK model parameterisation. From

[4]In this Scientific Opinion, the term kinetic data is used as general term referring to both ADME data (e.g. absorption rate, metabolic constant, etc.) as well as time-concentration data (e.g. plasma concentrations over time, time-dependent urinary excretion, etc.).

[5]In Europe, the term TRV is typically applied for reference values of pesticide active substances, i.e. acceptable daily intake (ADI), acute reference dose (ARfD), acceptable operator exposure level (AOEL) and acute acceptable operator exposure level (AAOEL). Therefore, TRV is used in this Scientific Opinion, but can also be read as health-based guidance value (HBGV).

here onwards, the term 'in vitro distribution kinetics' is used throughout the document instead of 'biokinetic studies' to avoid confusion.

- The Scientific Opinion will provide insight into the necessary studies to characterise the distribution of pesticide active substances within the 17 test systems used in the DNT IVB applying chemical-analytical and in silico approaches. This characterisation will help to translate nominal concentrations into biologically relevant in vitro exposure metrics for QIVIVE. Given that differences between nominal and actual cell exposure concentrations may vary significantly depending on test item, the cell culture material and cell culture media properties, the need for analytical measurements should be assessed to provide insight into the related uncertainty of the QIVIVE. The Scientific Opinion will provide recommendations for assessing in vitro distribution kinetics in DNT IVB assays.
- To determine the most relevant in vitro dose metric for QIVIVE, understanding the molecular target's location (e.g. tissue or organ) concerning the molecular initiating event (MIE) could guide this assessment. However, as such information is often unavailable, different in vitro dose metrics may need to be considered for QIVIVE based on potential target locations.

ToR 3: Defining a preferred approach for performing and reporting QIVIVE

- The Scientific Opinion will establish a standardised framework for conducting QIVIVE of DNT IVB data using PBK modelling, tailored for the hazard and risk assessment of pesticide active substances in the EU. This includes developing a reporting template covering the following key aspects: (a) PBK model selection and parameterisation, (b) evaluation of model simulations, (c) selection of appropriate in vitro dose metric and (d) assessment of QIVIVE outcomes, including uncertainty characterisation.
- Existing guidance on PBK model evaluation (EMA, 2018; FDA, 2018; OECD, 2021; WHO, 2010) will be referenced and, if necessary, adapted for the QIVIVE of DNT IVB data. Additionally, OECD recently published a document on general QIVIVE principles proposing a tiered approach to QIVIVE based on PBK model complexity (OECD, 2025), but it does not consider chemical-specific kinetic data requirements and uncertainty analysis. The Scientific Opinion will fill this gap and will provide a framework tailored for regulatory applications for pesticide active substances in the EU.
- The PPR Panel considers that both rats and humans are relevant for the QIVIVE of this work based on the following reasons:
    - The DNT IVB includes 13 human-based and 4 rat-based assays, making it relevant to perform QIVIVE for both species.
    - In vivo kinetic data for rats are generally available for pesticide active substances due to regulatory data requirements (e.g. OECD TG 417) and may inform PBK model parameterisation and evaluation.
    - Comparative in vitro metabolism data are often available for rats and humans because of regulatory data requirements and these may support PBK model development.
    - Oral equivalent doses obtained from QIVIVE can be compared to NOAELs, LOAELs and BMDLs from animal studies, enhancing hazard and risk assessment.
    - For humans, PBK models are crucial for assessing species differences in toxicokinetics; however, due to the scarcity of human in vivo kinetic data for pesticides, these models will rely mainly on in vitro and in silico data, which may lead to higher uncertainty in QIVIVE outcomes.

ToR 4: Defining key sources of uncertainty in QIVIVE for pesticide risk assessment

- The Scientific Opinion will identify the major sources of uncertainty in QIVIVE for DNT IVB data using PBK modelling and will propose a framework for identifying, describing and reporting these uncertainties. It will not define acceptable uncertainty levels as these are case-dependent and determined by risk assessors. Since EFSA recommends integrating DNT IVB data within an AOP-informed IATA framework, this Scientific Opinion will define the approach to assess QIVIVE uncertainty within this framework while acknowledging that data availability may differ across cases.
- The Scientific Opinion will provide recommendations on how to conduct and report uncertainty analyses, aligning with EFSA guidance documents on uncertainty analysis (EFSA Scientific Committee, 2018a) and use of weight of evidence (EFSA Scientific Committee, 2017a). The OECD guidance on characterisation, validation and reporting of PBK models for regulatory purposes (OECD, 2021) will also be considered.
- The Scientific Opinion will not address uncertainties related to the biological relevance and completeness of the DNT IVB itself (e.g. whether it sufficiently captures all relevant modes of action for DNT) since these aspects are already covered in the OECD's *Initial Recommendations on evaluation of data from the DNT IVB* (OECD, 2023a).
- The DNT IVB data available and/or currently generated within EFSA-funded projects for pesticide active substances[6] focus on the parent chemicals. Accordingly, the recommended QIVIVE approach will be based on data for the substance as tested in the DNT IVB assays. Although the test systems in the DNT IVB are generally not characterised for their

[6]https://ec.europa.eu/info/funding-tenders/opportunities/portal/screen/opportunities/tender-details/13967.

metabolic capacity, cell-based test systems typically lack metabolic activation systems and thus are assumed to have minimal metabolic conversion capacity. If toxicity data are only available for the parent chemical, the Scientific Opinion will address the resulting uncertainties regarding potential contributions of metabolites to DNT. It is, however, outside the scope of this document to propose a testing strategy for parent chemicals and their metabolites.

The four ToRs were translated into the following key assessment questions:

A What set of chemical-specific input parameters are required to parameterise a PBK model for QIVIVE of DNT IVB data, and what quality criteria should be applied to kinetic studies used to obtain these parameters?
B What studies are necessary to assess in vitro distribution kinetics to adequately translate the nominal concentrations used in the DNT IVB assays into relevant in vitro exposure metrics for QIVIVE?
C What is the preferred approach for conducting and reporting the QIVIVE of DNT IVB data for hazard and risk assessment of pesticide active substances in the EU, specifically regarding the following elements: (a) selection of the PBK model; (b) selection and evaluation of PBK model parameter values; (c) evaluation of the PBK model simulations; (d) selection of the vitro exposure metric(s) for translating internal to external exposure; (e) selection of the internal in vivo exposure metric(s) to correlate to the in vitro exposure metric(s); and (f) evaluation of QIVIVE outcomes?
D What is the preferred approach for conducting and reporting the uncertainty analysis of the proposed QIVIVE for pesticide human health hazard and risk assessment?

A protocol was established for this self-task mandate (Annex A), following the guidance on protocol development for EFSA generic scientific assessments (EFSA Scientific Committee, 2023). As indicated in the protocol, a systematic assessment of existing QIVIVE studies in the scientific literature was not expected to fully meet the requirements for the use of QIVIVE in regulatory hazard and risk assessment, and an expert knowledge-based approach, bringing together experts in the field of chemical risk assessment, mammalian toxicology (including DNT), in vitro toxicology, toxicokinetics, in vitro exposure metrics, PBK modelling and QIVIVE, was deemed more relevant. To reduce expert bias in the Scientific Opinion, a public consultation of the document is considered essential. Stakeholders' feedback (Annex G) received during the public consultation[7] on the draft output was considered for the finalisation of this Scientific Opinion. As indicated in the protocol (Annex A) focussed data collections were used when considered relevant by the WG. Two focussed data collections were performed, following the methodologies described in the related annexes (Annexes C and D).

1.3 | Additional information

This Scientific Opinion describes a recommended approach regarding the performance and reporting of QIVIVE of in vitro toxicity data obtained from the DNT IVB for pesticide active substances. In Section 1.3.1 a short description is provided of the assays and test systems that comprise the DNT IVB. It is noted that the assays and test systems of the DNT IVB are described in detail in the OECD document *Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In Vitro Battery (IVB)* (OECD, 2023a). That document also provides initial recommendations on the evaluation of data developed with the DNT IVB (e.g. hit vs. non-hit, uncertainties, biological coverage). The major aims formulated in the document include description of the assays that comprise the battery in terms of neurodevelopment, and provision of criteria that allow evaluation of the relevance of the data to DNT and that assist in the determination of the degree of certainty in any positive or negative findings to better inform use of DNT in vitro data in regulatory hazard determinations. The document was approved by the OECD Working Party of the National Coordinators of the Test Guidelines Programme at its 35th meeting in April 2023 and was most recently updated in November 2023.

Additionally, as an activity of the OECD Expert Group on DNT IVB, the document *Principles of quantitative in vitro to in vivo extrapolation (QIVIVE) – As example applied to the DNT IVB* (OECD, 2025) was developed. In Section 1.3.2 a short description of this document, which serves as the basis for the development of this Scientific Opinion, is provided.

1.3.1 | DNT IVB assays

The information presented here reflects the 17 assays of the DNT IVB described in the abovementioned OECD document (OECD, 2023a).

For the establishment of the DNT IVB, in vitro test systems based on human or rat cells with selected readouts have been put together to allow study of the possible disturbance of key neurodevelopmental processes by chemicals. The key neurodevelopmental processes covered and the related assays in the DNT IVB are:

1. Neuroprogenitor cell proliferation: two assays (*NPC1* and *hNP1 Prolif*) based on two human cell-based test systems.
2. Neuroprogenitor cell apoptosis: one assay (*hNP1 Apop*) based on a human cell-based test system.
3. Cell migration

[7] https://connect.efsa.europa.eu/RM/s/consultations/publicconsultation2/a0ITk000004vWyaIAE/pc1517.

a. Neural stem cells: One assay (*UKN2*) based on a human cell-based test system.
b. Radial glia cells: One assay (*NPC2a*) based on a human cell-based test system.
c. Neuronal cells: One assay (*NPC2b*) based on human cell-based test system.
d. Oligodendrocyte cells: One assay (*NPC2c*) based on human cell-based test system.

4. Neuroprogenitor cell differentiation to neurons: one assay (*NPC3*) based on a human cell-based test system.
5. Neurite outgrowth.

a. Neurons: three assays (*NPC4*, *UKN4* and *hN initiation*) based on three human cell-based test systems and one assay (*Cortical Initiation*) based on a rat cell-based test system.
b. Peripheral neurons: one assay (*UKN5*) based on a human cell-based test system.

6. Neurite maturation: one assay (*Cortical Maturation*) based on a rat cell-based test system.
7. Synaptogenesis: one assay (*Cortical Synapto*) based on a rat cell-based test system.
8. Neuroprogenitor cell differentiation to glial cells: one assay (*NPC5*) based on a human cell-based test system.
9. Neural network formation: one assay (*Cortical MEA*) based on a rat cell-based test system.

1.3.2 | OECD document QIVIVE DNT IVB

The OECD document *Principles of quantitative in vitro to in vivo extrapolation (QIVIVE) – As example applied to the DNT IVB* (OECD, 2025) provides an overview of QIVIVE principles, particularly through the application of PBK modelling, to facilitate the incorporation of DNT IVB data into chemical assessment. The document was developed as part of an activity by the OECD Expert Group on DNT IVB and is also one of the deliverables of the OECD's Working Party on Hazard Assessment (WPHA) project on the 'Development of an IATA Framework Template for Developmental Neurotoxicity (DNT) and relevant Guidance'. The Expert Group on DNT IVB reviewed the document in September 2023 and the WPHA reviewed the document in October/November 2024 and in April/May 2025. The document was revised accordingly and eventually adopted by the WPHA in June 2025.

The OECD document focuses on introducing the general concept of QIVIVE for integrating DNT IVB data into chemical assessment. It is not intended as a technical guide for conducting or evaluating a QIVIVE analysis and is not tailored to a specific regulatory context. Possible applications of QIVIVE covered in the OECD QIVIVE document range from chemical screening to contextualisation of DNT IVB data in hazard and risk assessment. This Scientific Opinion used this OECD document as a basis but tailored it to hazard and risk assessment of pesticide active substances in the EU.

2 | INTRODUCTION TO QIVIVE AND OVERVIEW OF THE STRUCTURE AND KEY POINTS OF THE SCIENTIFIC OPINION

2.1 | Introduction to QIVIVE

The translation of in vitro exposures to related in vivo external exposures was first reported in the scientific literature in 1999 (Dejongh et al., 1999), and the term 'quantitative in vitro to in vivo extrapolation' (QIVIVE) was introduced later by Yoon et al. (2012). The authors defined QIVIVE as '*the process of estimating the **environmental exposures** to a chemical that could produce **target tissue exposures** in humans equivalent to those associated with effects in an in vitro toxicity test (e.g. an EC50, a benchmark concentration or an interaction threshold identified by a biologically based dose–response model for the toxicity pathway of concern)*'. An 'environmental exposure' can be considered as any external exposure and is often called an 'external equivalent dose'. Furthermore, besides for humans, QIVIVE can also be performed for animals. Typically, PBK modelling[8] is the tool that has been used to translate an external exposure to a target tissue exposure (Blaauboer, 2001, 2003, 2010; Chang et al., 2022; Louisse et al., 2017; Yoon et al., 2012). QIVIVE approaches based on PBK modelling use a forward or reverse dosimetry approach and are mainly focussed on oral exposure, thereby providing an oral equivalent dose (OED).

With a forward dosimetry approach, a PBK model is used to estimate the internal exposure (e.g. a plasma or a target tissue concentration) from a given external exposure scenario. The estimated internal exposure is then compared to (no) effect concentrations from an in vitro toxicity assay of interest in order to quantitatively contextualise in vitro toxicity data, i.e. the margin between the estimated internal concentration and the in vitro concentration of interest is determined. External exposure selected as starting point can, for example, be an available TRV or a given exposure estimate (Figure 1A).

Conversely, with a reverse dosimetry approach, a PBK model is used to estimate the external exposure from a given internal exposure. The internal exposure used as the starting point for the QIVIVE can be based on an effect concentration (e.g. a given benchmark concentration (BMC)) or a no effect concentration (NOEC) from one or more in vitro toxicity assays

[8]In this document, the term physiologically based kinetic (PBK) modelling is used, which can also be read as physiologically based pharmacokinetic (PBPK), physiologically based toxicokinetic (PBTK), or physiologically based biokinetic (PBBK) modelling.

of interest. To quantitatively contextualise in vitro toxicity data, the estimated external exposure is compared to a relevant external exposure metric, such as a TRV or a specific exposure estimate, thus defining the margin between the estimated external exposure and the reference value or actual exposure value (Figure 1B). In various proof-of-principle studies, obtained OEDs have been compared to NOAELs/LOAELs/BMDLs obtained from animal studies, showing in most cases that OEDs are within an order of magnitude with other reference points[9] (see, e.g. Chen et al., 2018; Dejongh et al., 1999; Li et al., 2017; Louisse et al., 2010; Omwenga et al., 2021; Strikwold et al., 2013; Verwei et al., 2006). No such proof-of-principle studies have been published in which OEDs for in vitro DNT effects have been compared with reference points from in vivo DNT studies.

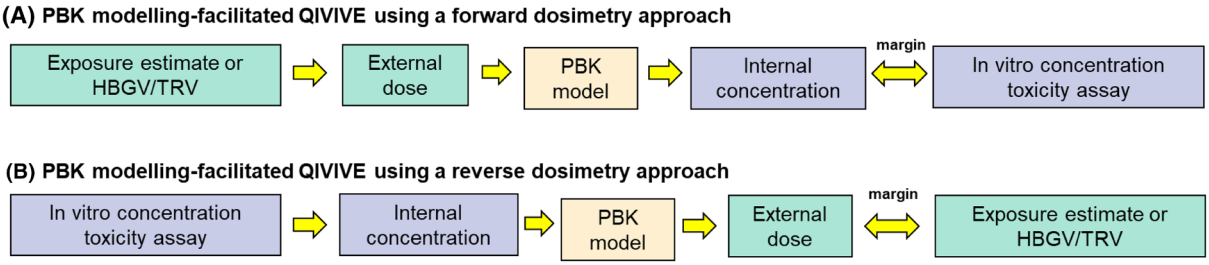

FIGURE 1 Schematic representation of PBK modelling-facilitated QIVIVE using a forward dosimetry approach (A) or a reverse dosimetry approach (B).

The principles of forward and reverse dosimetry-based QIVIVE are largely similar, with the primary distinction being the starting point of the assessment (i.e. external exposure for forward dosimetry and internal exposure for reverse dosimetry). In reverse dosimetry QIVIVE methods, the PBK model itself is generally applied in a forward dosimetry manner, predicting a range of internal exposures based on a range of external exposures, as illustrated in Figure 2B. An OED is then obtained from the internal concentration that is aligned to the in vitro concentration. In both forward and reverse dosimetry QIVIVE approaches, a link is made between an in vitro concentration and an in vivo exposure (external dose). Also, for both approaches a link needs to be made between the internal exposure metric and the concentration applied in the in vitro toxicity test (see Section 4).

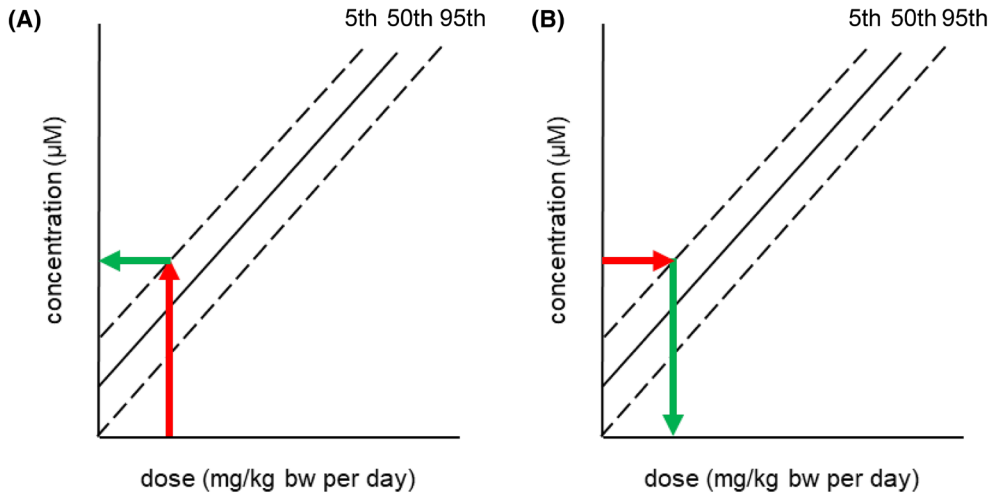

FIGURE 2 PBK modelling-facilitated QIVIVE, linking external exposure to internal exposure, using a forward dosimetry approach (A) or a reverse dosimetry approach (B). The graphs show the PBK modelling-based description of the relation between external exposures (*X*-axis, dose in mg/kg bw per day) and internal exposures (*Y*-axis, e.g. C_{max} in plasma). In this hypothetical example, the toxicokinetics are linear in the given exposure range and the PBK model describes inter-individual differences in toxicokinetics, providing internal exposure estimates for the median of a given population (straight line) and the 5th and 95th percentile of the population (dashed lines). The starting point of the QIVIVE using a forward dosimetry approach (A) is an external dose (red arrow), resulting in a related internal concentration (green arrow). The starting point of the QIVIVE using a reverse dosimetry approach (B) is an internal concentration based on data from an in vitro toxicity assay (red arrow), resulting in a related external dose (green arrow).

Various QIVIVE examples have been described in the scientific literature, ranging from high throughput approaches to prioritise (environmental) chemicals for more in-depth risk assessment (e.g. Bell et al., 2018; Rotroff et al., 2010; Wetmore

[9]A reference point is a dose derived from an experimental/observational dose–response relationship (e.g. BMDL/NOAEL/LOAEL) that reflects the critical toxicological effect (EFSA Scientific Committee, 2025). Reference point can also be read as point of departure (PoD). In the context of this Scientific Opinion, reference point is also used in certain cases when not necessarily relating to the critical toxicological effect.

et al., 2012), to lower throughput approaches providing evidence that QIVIVE of in vitro toxicity data may be used to derive reference points for the hazard and risk assessment of chemicals (e.g. Chen et al., 2018; Dejongh et al., 1999; Li et al., 2017; Louisse et al., 2010; Omwenga et al., 2021; Strikwold et al., 2013; Verwei et al., 2006).

Despite the availability of the cited QIVIVE examples, there is, so far, limited application of QIVIVE in regulatory hazard and risk assessments. Within the pesticide assessment in the EU, two QIVIVE examples have been evaluated by EFSA and/or Member States. In the peer review of the active substance deltamethrin, a QIVIVE approach submitted by the applicant was evaluated and related uncertainties were identified.[10] For acetamiprid, a dedicated EFSA PPR Panel WG was established to evaluate available in vitro and in vivo evidence on DNT. This WG assessed whether in vitro DNT data could be extrapolated to external equivalent doses using PBK modelling-facilitated QIVIVE. No QIVIVE was performed, given that the available kinetic data were too limited to parameterise a PBK model for the QIVIVE (EFSA, 2024). Furthermore, for the quantitative interpretation of in vitro data on mitochondrial effects in the brain, QIVIVE was performed in an EFSA-funded project on the applications of NAMs for the risk assessment of tebufenpyrad (Henri et al., 2022). This study is being evaluated by an EFSA WG on neurotoxicity,[11] providing important insights into application and related uncertainties of QIVIVE approaches for regulatory hazard and risk assessment.

2.2 | Overview of the structure and key points of the Scientific Opinion

The aim of this Scientific Opinion is to develop a framework for performing and reporting QIVIVE of data from the DNT IVB for the hazard and risk assessment of pesticide active substances in the EU. The PPR Panel already recommended using an (AOP-informed) IATA for the interpretation of DNT IVB data (EFSA PPR Panel, 2021a), and QIVIVE is to be integrated in such an approach. In the presence of in vivo DNT data, PBK modelling-facilitated QIVIVE can be used for the dose concordance analysis of the available in vitro and in vivo data. In the absence of in vivo DNT data, PBK modelling-facilitated QIVIVE can be used to gain insights how the available reference points (related to other endpoints than DNT) for the active substance under evaluation compare to internal exposure that is associated with effects in the test systems of the DNT IVB.

PBK modelling-facilitated QIVIVE entails various steps, with each step having its critical aspects to consider. These different steps are reflected in the ToRs of this self-task mandate and are discussed in detail in this Scientific Opinion.

In the following, the topics of the Scientific Opinion are shortly introduced, also linking to the ToRs of the mandate.

Section 3 provides key analytical considerations for experiments supporting QIVIVE. These are relevant for in vitro distribution kinetic studies of the test item in the DNT IVB assays (Section 4), as well as for in vitro and in vivo kinetic studies required for PBK model parameterisation/evaluation (Section 5). This section provides information to address ToRs 1 and 2.

Section 4 discusses how a concentration from an in vitro toxicity assay can be linked to an internal in vivo concentration for use in the PBK model within the QIVIVE framework. In the scientific literature, different approaches have been applied for this aspect of QIVIVE, but no standardised or harmonised approach is available. This section describes applied approaches in the scientific literature and provides proposals of those that are most common and considered most scientifically sound. This section provides information to address ToRs 2 and 3.

Section 5 discusses PBK modelling, including the selection of the PBK model for QIVIVE based on the tiered PBK modelling strategy proposed in OECD (2025). It provides insight into the data sources and methods relevant for the generation of chemical-specific PBK model parameter values, considering in vivo, in vitro and in silico approaches. Given that only very few test guideline methods are currently available to provide data for PBK model parameterisation, the PPR Panel considers it of utmost importance that kinetic studies are well designed and critically assessed in order to provide reliable data for PBK model parameterisation and/or evaluation. This section aims to provide some insight into aspects of well-conducted (in vitro) kinetic studies, from which the outcomes can be used by those that conduct or evaluate the QIVIVE. Besides analytical aspects (Section 3), other aspects, such as test system characterisation, use of reference chemicals in the test systems, assessment of in vitro distribution kinetics, application of acceptance criteria and possible need for chemical-specific optimisation of the experimental conditions, are discussed in this section. This section provides information to address ToRs 1 and 3.

Section 6 provides considerations related to human and animal metabolites. The test systems of the DNT IVB are considered to have no/very limited biotransformation capacity, and no information is available whether the assays of the DNT IVB are compatible in co-culture with biotransformation systems, such as S9. Therefore, in order to have insight into the toxicity of human and animal metabolites in the DNT IVB, these would need to be tested as such. This section provides information to address ToR 4.

Section 7 provides an overview of the main aspects to be considered in the uncertainty analysis related to the QIVIVE of DNT IVB data for the hazard and risk assessment of pesticide active substances. This section provides information to address ToR 4. It should be highlighted that acceptable levels of uncertainty are not defined here as these are case-dependent and determined by risk assessors.

[10]The peer review of deltamethrin is still ongoing and therefore, only limited information on the assessment is currently publicly available. Limited information can be found in the public minutes of the peer review meeting on deltamethrin (TC 110): https://www.efsa.europa.eu/sites/default/files/2023-12/minutes-ppr-mammalian-toxicity.pdf.

[11]https://www.efsa.europa.eu/sites/default/files/2025-03/1st%20WG%20Neurotoxicity%20minutes.pdf.

Section 8 presents a framework of performing and reporting QIVIVE of DNT IVB data for the hazard and risk assessment of pesticide active substances, considering (1) the selection of the in vitro exposure metric, (2) the PBK modelling, (3) the translation of the in vitro exposure metric to the external equivalent in vivo dose and (4) the uncertainty analysis. A reporting template for this is available as Annex F to this Scientific Opinion. This section provides information to address ToRs 3 and 4.

Section 9 provides conclusions relating to the four ToRs.

Section 10 provides recommendations on the QIVIVE of in vitro toxicity data from the DNT IVB for the hazard and risk assessment of pesticide active substances, which are able to improve the in vitro testing output interpretation, the QIVIVE approach as well as the PBK modelling.

3 | ANALYTICAL CONSIDERATIONS

This section outlines key analytical aspects to consider for experiments to be performed for QIVIVE. Such studies include studies on in vitro distribution kinetics of test items (Section 4) as well as in vitro and in vivo kinetic studies that provide data for PBK model parameterisation/evaluation (Section 5). Currently, no guidance or test guidelines are available for such studies although some relevant information is provided in the OECD Good In vitro Method Practice (GIVIMP) document (OECD, 2018). Since adequate analytical methods are key to obtain reliable data, this section provides information on key analytical aspects to support those generating data for or evaluating the QIVIVE.

Analytical assessment is key for various aspects of the approval process of pesticide active substances and for monitoring of pesticide residues. Valid and reliable analytical methods are required for accurate quantification of the test material applied in the toxicity studies and for pesticide residue analysis.

Analytical methods are required for the following:

- The analysis of plant protection products, including active substances, relevant impurities and co-formulants.
- The determination of pesticide residues in various matrices, such as soil, water, sediment, air, food, feed and body fluids and tissues.

Metabolites are included in the analytical scope if they are part of the residue definition[12] established for risk assessment or monitoring purposes.

To ensure specificity and reliability, analytical methods must be properly validated and supported by confirmatory techniques, when appropriate and submitted to regulatory authorities as part of the approval dossier. Commission Regulation (EU) No 284/2013 provides detailed requirements for the analysis of active substances, relevant impurities and residues in various matrices. While this regulation primarily supports the approval process for plant protection products, its provisions on analytical method validation can inform the design of analytical methods used in studies that support the QIVIVE.

This section focusses on relevant aspects of the analytical methods and performance criteria drawing primarily on guidance related to the analysis of pesticide residues and metabolites in food commodities and human samples (e.g. EC, 2023), where such guidance exists and/or is being developed (Section 3.1), addresses general principles of method validation and quality control (Section 3.2) and discusses analytical considerations specific to in vitro distribution studies and kinetic studies relevant for PBK model parameterisation needed for QIVIVE applications (Section 3.3). Some more background on monitoring of pesticide residues and metabolites in food commodities and human samples is provided in Appendix A.

3.1 | Analytical methodologies for pesticide residue determination

3.1.1 | Extraction techniques

Sample preparation is a critical step in the analytical workflow with the choice of extraction technique playing a key role in isolating pesticides and their metabolites from complex matrices. The main challenges include maximising analyte recovery while minimising matrix interferences and preventing contamination. Techniques, such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE), are used to isolate pesticides and their metabolites from the prepared samples.

Common extraction techniques include quick, easy, cheap, effective, rugged, and safe (QuEChERS), SPE and LLE. QuEChERS is preferred for its simplicity and efficiency in extracting a wide range of pesticide residues from diverse matrices using acetonitrile, followed by a cleanup step with dispersive SPE. SPE, in turn, uses solid adsorbents to isolate, concentrate and purify analytes, providing high selectivity and effectiveness for complex samples. LLE separates analytes based on their solubility in different solvents, making it particularly suitable for non-polar pesticides. Each technique must be

[12]In the context of pesticide risk assessment, the residue definition identifies the chemical entities (such as the parent chemical, its metabolites, degradation and reaction products) that are relevant for monitoring and for the evaluation of dietary exposure. This includes only those components that are toxicologically significant, as they may pose a risk to consumers. The residue definition is essential for determining which chemicals should be measured in food and feed commodities for regulatory and risk assessment purposes. This approach is supported by EFSA (EFSA PPR Panel, 2016), which provides detailed guidance on how to establish residue definitions for dietary risk assessment, ensuring that all relevant chemicals contributing to the overall toxicological burden are appropriately considered.

optimised and validated for the specific matrix and target analytes to ensure reproducibility and compliance with regulatory performance criteria (EC, 2023).

3.1.2 | Instrumental analysis of pesticide residues

After extraction, instrumental analysis is essential for the identification and quantification of pesticides, including their metabolites and residues. The most commonly used techniques are gas chromatography (GC) and liquid chromatography (LC), both typically coupled with mass spectrometry (MS) for enhanced specificity and sensitivity. Chromatographic methods separate complex mixtures of analytes based on their chemical properties, allowing for quantification when appropriate analytical standards are available. MS further identifies and quantifies analytes by measuring their mass-to-charge ratios, providing structural information that enhances analytical confidence.

GC–MS is particularly effective for analysing volatile and semi-volatile chemicals, providing excellent sensitivity and specificity, but often requires derivatisation of non-volatile or thermally labile chemicals to make them amenable to GC analysis. LC–MS is preferred for polar, non-volatile and thermally labile chemicals due to its broader analyte range and superior quantification capabilities (EC, 2023). LC–MS generally requires less sample preparation than GC–MS, as derivatisation is usually not necessary. LC–MS techniques provide higher sensitivity, lower detection limits and better handling of matrix effects, enabling more accurate and efficient detection of trace pesticides residues and metabolites in complex samples.

To further enhance analytical performance, tandem and multi-stage MS techniques are employed. LC–MS uses a single MS stage, while LC–MS/MS (tandem MS) incorporates a second mass analyser for ion fragmentation and structural elucidation, increasing selectivity and minimising background interference. LC–MS/MS/MS (triple quadrupole MS or QqQ) incorporates a third fragmentation stage, significantly improving sensitivity, selectivity, background noise reduction and the quantification of low-abundance analytes in complex mixtures (Kailasam, 2024).

These methods are primarily applied to non-radiolabelled chemicals, which is typical for pesticide residue analysis in food commodities and biological samples. For radiolabelled chemicals used in controlled-exposure studies such as in vitro comparative metabolism and ADME studies, chromatographic separation is combined with radioactivity detection techniques to quantify radiolabelled substances. MS can be used in parallel for structural identification and quantification of metabolites, but it does not directly detect the radioactive label. Analytical approaches focusing specifically on radiolabelled pesticides and their application in studies supporting QIVIVE are further detailed in Section 3.3.

3.1.3 | Use of internal standards in quantitative analysis

An Internal Standard (IS) is a chemical added at a consistent concentration to all samples, including calibration standards, during quantitative chromatographic analysis. The analyte concentration is determined by calculating the ratio analyte's peak/IS' peak, which corrects for variations in sample preparation, injection volume and instrument response as the IS is affected similarly as the target analytes. The use of IS reduces both random and systematic errors, improves precision and minimises the need for repeat analyses.

Selecting an appropriate IS requires choosing a chemical that does not co-elute or interfere with analytes or matrix components. Ideally, the IS should resemble the target analyte in behaviour, retention time, peak shape and detector response. A common practice in GC–MS is to use isotopically labelled analogues (e.g. deuterated forms) of the analyte as IS. The IS should be introduced at the same concentration across all samples, ideally close to the concentration of the target analytes. Incorporating the IS early in the sample preparation helps account for variability throughout the entire analytical process. For multi-analyte methods, multiple IS may be used to ensure accurate quantification across chemically diverse chemicals.

3.1.4 | Challenges in pesticide residue analysis

Pesticide residue analysis faces several significant analytical challenges, particularly when dealing with complex matrices that contain interfering substances. Meeting the stringent low detection limits required for regulatory compliance adds further complexity to the analytical workflow. The wide variety of pesticides, each with different chemical properties, requires multiple complementary analytical methods. Efficient extraction and purification techniques must be optimised to maximise analyte recovery while minimising loss and contamination. Developing and validating accurate and reproducible analytical methods is essential but resource intensive.

In addition, evolving regulatory requirements and international standards demand continual method adaptation to ensure compliance. The reliance on advanced, often costly instrumentation, such as LC–MS/MS, can limit access to pesticide residue analysis for some laboratories, posing challenges for widespread monitoring and enforcement.

3.2 | Validation and quality control of analytical methods

3.2.1 | Performance criteria for method validation

Method validation and analytical quality control (AQC) are critical components for ensuring the accuracy, reliability and regulatory compliance of pesticide residue analysis in food, feed and biological samples. A validated method demonstrates that it is suitable for its intended purpose, while ongoing quality control ensures consistent performance over time. Laboratories validate methods through structured steps to confirm their suitability for the intended purpose.

Method validation follows a structured, stepwise approach. The first step is defining the analytical purpose, which involves understanding the target analytes, the relevant sample matrix and the required sensitivity and specificity. Key variables that could affect method performance, such as temperature, pH, analyte concentration and matrix composition, are identified and considered in method development. Appropriate samples are selected to represent the range of expected real-world conditions under which the method will be used. Finally, analytical performance is evaluated using predefined criteria to ensure the method is robust, reproducible and accurate.

Key performance criteria of a method typically include (EC, 2023):

- Specificity: the method's ability to accurately detect, identify and quantify the analyte without interference from other substances, such as metabolites, degradation products or matrix constituents.
- Linearity: the method's ability to produce results directly proportional to the expected concentration of the analyte within a given range.
- Accuracy: how close the measured results are to the true or accepted reference value.
- Precision: the degree of repeatability (intra-day) and reproducibility (inter-day, inter-operator) of the analytical results.
- Recovery: the proportion of the analyte that is successfully extracted and quantified from the sample.
- Limit of detection (LOD) and limit of quantification (LOQ): the lowest concentration of analyte that can be reliably detected and quantified, respectively.

Statistical analysis of the validation data is essential to determine the method's performance and robustness. All validation activities and results are thoroughly documented, detailing the methods used, results obtained and any deviations from the expected outcomes. The validated method is then reviewed and approved by qualified personnel to confirm that the method is fit for its intended use.

3.2.2 | Analytical quality control (AQC)

After method validation, laboratories must implement continuous quality control (QC) procedures to monitor method performance over time. QC practices ensure that analytical results remain reliable, reproducible and within acceptable limits.[13] QC tools include calibration curves, matrix-matched standards, internal standards and control samples (including procedural blanks, spiked samples and quality control reference materials) (FAO/WHO, 2017), which help compensate for matrix effects and improve quantification accuracy. LOD, LOQ and recovery rates must be rigorously evaluated to confirm sustained method reliability.

By following these steps, laboratories can ensure their analytical methods are validated and capable of producing reliable and consistent results.

3.2.3 | Pesticide analytical methods for risk assessment

The 'Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes' from the European Commission (EC, 2023) provides comprehensive instructions for the validation and application of analytical methods in pesticide residue analysis. This document, which supersedes the previous SANCO/3029/99 and SANCO/825/00 guidelines, aims to harmonise practices and enhance the accuracy, reliability and consistency of pesticide residue analysis. It addresses key aspects, such as method validation, matrix effects and the use of advanced analytical techniques, ensuring that laboratories can effectively monitor and control pesticide levels in various matrices. Principles described in the guidance document are considered of relevance for analytical methods applied for QIVIVE.

Section 5 of the EC Guidance Document details the validation requirements for methods used in post-approval control and monitoring of pesticides. Section 5.6 of that document specifically addresses analytical methods for monitoring residues in body fluids and tissues, emphasising the need for reliable and reproducible methods suitable for their intended purpose and consistent across laboratories and over time. The section highlights the importance of stringent quality

[13]Common guidelines for the quality control (QC) of analytical samples state that recovery and repeatability are considered acceptable if mean recoveries at each concentration level is 70%–120% (ICH guideline Q2(R2) on validation of analytical procedures https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q2r2-validation-analytical-procedures-step-2b_en.pdf) and the precision ≤ 20% coefficient of variation (https://sisu.ut.ee/lcms_method_validation/41-precision-trueness-accuracy/).

control measures to maintain high analytical performance standards. Key aspects highlighted in that section include (a) selecting analytical methods appropriate for the sample matrix and target residues; (b) implementing QC through regular checks, internal standards and control samples; (c) documenting of the validation process to ensure regulatory compliance and reproducibility.

Matrix considerations are crucial as different biological fluids and tissues may require specific preparation and extraction techniques to minimise matrix effects that could interfere with residue detection. Adhering to these guidelines supports robust, reliable and compliant pesticide residue analyses, ultimately contributing to public health and environmental protection.

3.3 | Analytical requirements for QIVIVE studies

As mentioned earlier, no guidance or test guidelines are currently available for evaluating in vitro distribution or for conducting kinetic studies that support the parameterisation and evaluation of PBK models. Therefore, accurate and reliable QIVIVE requires specific attention to the selection and implementation of robust analytical methodologies. Two key analytical needs are: (1) quantification of pesticides and their metabolites in ADME studies using both in vitro and in vivo test systems and (2) characterisation of chemical distribution in in vitro test systems, particularly for deriving toxicokinetic parameters and toxicodynamic endpoints.

While analytical methods for the assessment of pesticides and their metabolites in food commodities and human samples offer relevant principles (see Sections 3.1 and 3.2), adaptations are needed for their application to in vitro distribution studies or for kinetic studies for PBK model parameterisation/evaluation.

To support reliable QIVIVE and PBK model development, the following analytical aspects must be addressed:

a. **Sample preparation and matrix effects**. Efficient extraction methods like SPE or LLE are essential for isolating pesticides from complex in vitro media. Addressing matrix effects, such as interference from medium components, is necessary to maintain analytical accuracy. To minimise matrix effects, calibration curves should be developed using standards prepared in the same medium (Wu & Zou, 2020). The use of internal standards, particularly isotopically labelled compounds, enhances analytical accuracy by compensating for potential losses or variability during sample preparation and analysis (Reddy, 2017).
b. **Analytical techniques and validation**. For the sensitive and selective detection of pesticides, techniques such as LC–MS/MS or GC–MS are preferred, given their ability to accurately quantify even trace levels. These methods require rigorous validation for parameters such as accuracy, precision, LOD and LOQ to ensure data reliability (Romaniou et al., 2022). These techniques are well-suited for the analysis of non-radiolabelled chemicals. In the case of radiolabelled chemicals, they are also applicable; however, complementary radioactivity quantification is needed to determine total concentrations of the parent chemicals and its metabolites. In regulatory in vivo ADME studies, the use of radiolabelled chemicals enables the simultaneous quantification of parent chemicals and their metabolites by measuring total sample radioactivity (see Section 5.7.3 for more details). For comparative in vitro metabolism studies of pesticide active substances, the use of radiolabelled test materials is recommended, as outlined in a previous EFSA PPR Panel Scientific Opinion (EFSA PPR Panel, 2021b). That Opinion proposed a complementary analytical strategy, combining radioactivity detection with UV absorption and/or mass spectrometry. Radiolabelled test materials are typically available for pesticide active substances, as they are routinely used in regulatory ADME and metabolism studies. Their availability supports integration into PBK modelling workflows. Accordingly, the PPR Panel recommends using this combined quantification strategy, based on separate but complementary analytical platforms, to enable robust recovery assessment and accurate compound tracking in controlled-exposure in vitro and in vivo kinetic studies.

These analytical considerations are essential for generating high-quality, reliable and interpretable kinetic data that support the derivation of in vitro exposure metrics (see Section 4), as well as the parameterisation and evaluation of PBK models (see Section 5.7 and 5.8). By integrating validated experimental data into modelling workflows (through robust analytical techniques) the kinetic behaviour of pesticides can be accurately characterised. This, in turn, provides a robust foundation for QIVIVE and the development of scientifically sound PBK models, which are critical for modern, mechanism-informed chemical risk assessment (OECD, 2021).

4 | LINKING IN VITRO EXPOSURE TO INTERNAL EXPOSURE METRICS

An important step of the QIVIVE is linking the exposure applied in the in vitro assays, e.g. (no) effect concentrations, to the internal exposure to be used in the PBK modelling. One needs to decide on the following elements: (1) whether the QIVIVE is performed based on a maximum (C_{max}) or steady-state (C_{ss}) concentrations or on the area under the concentration-time curve (AUC; the product of concentration and exposure time), (2) the in vitro exposure metric used (medium and cell-associated concentration) and (3) the in vivo internal exposure (plasma and tissue/organ).

The PPR Panel recommends focussing QIVIVE on C_{max} or C_{ss} concentrations in plasma. However, it finds it helpful to also consider QIVIVE based on other exposure metrics (e.g. AUC) and compare outcomes when analysing uncertainties (see Section 7.1.3), acknowledging that information to derive an in vitro AUC is mostly not available.

The PPR Panel further recommends linking the maximum unbound concentration in the exposure medium of the DNT IVB assay to the C_{max} or C_{ss} unbound plasma concentration in vivo in PBK models (Scenario A; Figure 3). For baseline cytotoxicity,[14] as can be assessed, for example, using the approach in Lee et al. (2022), QIVIVE may also be performed by linking the maximum cell-associated concentration in vitro to the C_{max} or C_{ss} tissue concentration in vivo (Scenario B; Figure 3). If no information is available on the maximum unbound and cell-associated concentration in vitro, the nominal concentration may be applied provided that the uncertainties associated with the use of this exposure metric are assessed (see Section 4.2).

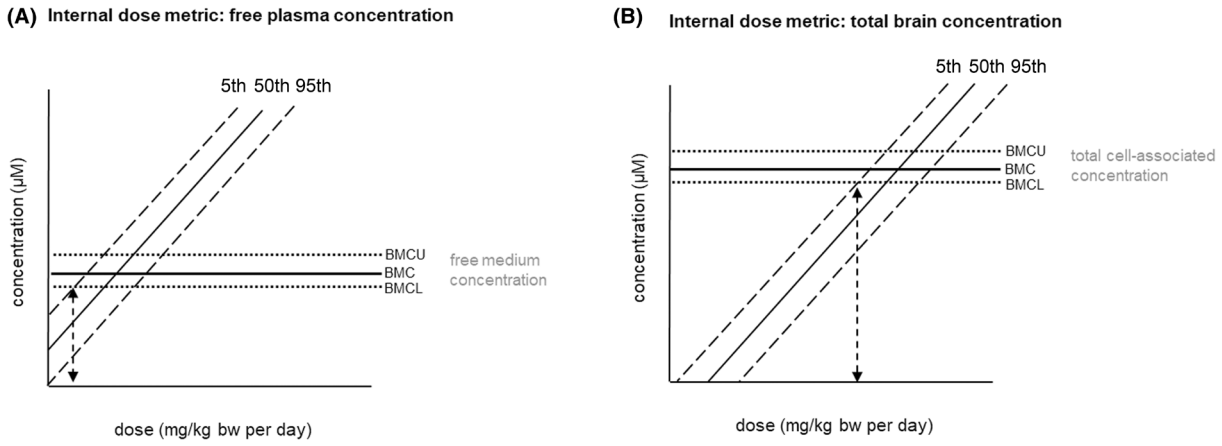

FIGURE 3 A hypothetical QIVIVE example demonstrating the linking of (A) the unbound medium concentration in vitro to the unbound concentration in plasma or (B) the total cell-associated concentration to the total tissue (e.g. brain) concentration. Here, the toxicokinetics are linear in the given exposure range and the PBK model describes inter-individual differences in toxicokinetics, providing internal exposure estimates for the median of a given population (solid line) and the 5th and 95th percentile of the population (dashed lines). QIVIVE is performed using a given in vitro toxicity assay, for which a selected benchmark response (BMR) is considered the relevant reference point, resulting in the corresponding benchmark concentration (BMC; horizontal solid line in bold) value with its upper and lower confidence limits (BMCU and BMCL, respectively; horizontal dotted lines). The OED obtained (represented by the arrow crossing the X-axis) from scenario A is lower than scenario B, but this is not necessarily always the case. It is noted that selection of the BMR can have great impact on calculations and should be robustly documented and justified.

The OECD QIVIVE document (OECD, 2025) recommends linking the unbound chemical concentration in medium in vitro to the unbound concentration in plasma for the QIVIVE, which is in line with the PPR Panel recommendation. In the OECD QIVIVE document, no considerations are provided of a relevant internal exposure metric to which cell-associated concentrations are to be linked to. The rationale for the recommendations by the PPR Panel is provided in Sections 4.1 and 4.2.

When performing QIVIVE one needs to realise that exposure duration in vitro is not directly comparable to the in vivo scenario. Most assays of the DNT IVB apply single administration (exposure duration of one to a few days), and some of the DNT IVB assays apply repeated administration over several days (OECD, 2023a). This is an inherent uncertainty that is to be considered in the uncertainty analysis (see Section 7.1.4).

4.1 | In vivo internal exposure metrics for QIVIVE

Currently, there is no harmonised approach on how an in vitro (effect) concentration should be linked to an in vivo internal exposure metric, and the most adequate approach may differ depending on chemical characteristics and/or the nature and location (e.g. tissue or organ) of the molecular interaction between the chemical and its target (Escher et al., 2011; Groothuis et al., 2015; Lee et al., 2022; Mielke et al., 2019; Rietjens et al., 2019). Several internal exposure metrics in vivo have been used in QIVIVE to link in vitro effect concentrations to, including the AUC, C_{max} or C_{ss} total or unbound concentration in plasma or tissue. Many published QIVIVE studies used in vitro effect concentrations (e.g. nominal, unbound in medium or cell-associated) and linked them to C_{max} or C_{ss} concentrations in blood, plasma or tissue in vivo (e.g. Chen et al., 2018; Dejongh et al., 1999; Li et al., 2017; Louisse et al., 2010; Omwenga et al., 2021; Strikwold et al., 2013; Verwei et al., 2006). Some studies also considered linking the AUC in vitro to an AUC in vivo (e.g. Fragki et al., 2023; Louisse et al., 2015). However, this latter approach may be less reliable, especially when in vitro exposure is much shorter than the modelled in vivo exposure period, also considering the difficulty in derivation of in vitro AUCs. The limited QIVIVE examples based on the AUC have yielded less conservative OED estimates than those based on C_{max} or C_{ss} concentrations in vivo (Fragki et al., 2023; Louisse

[14]Baseline cytotoxicity refers to general cell damage caused by a chemical's hydrophobicity. It reflects the minimum, non-specific toxicity resulting from a chemical's accumulation in the cell membrane, which disrupts the membrane's structure and function. In contrast, enhanced cytotoxicity indicates that a chemical is more likely to act through specific modes of action (MOA) on cell viability.

et al., 2015). Proof-of-principle studies that compared QIVIVE-based OEDs to in vivo reference points (e.g. NOAEL or BMDL) have reported reasonable concordance when C_{max} or C_{ss} concentrations in vivo were linked to in vitro effect concentrations (Chen et al., 2018; Dejongh et al., 1999; Li et al., 2017; Louisse et al., 2010; Omwenga et al., 2021; Strikwold et al., 2013; Verwei et al., 2006). Furthermore, Daston et al. (2010) proposed a validation list of control chemicals for developmental toxicity testing based on maternal plasma C_{max}, supporting plasma C_{max} as a relevant internal exposure metric in this context. Based on these considerations, linking the in vitro exposure concentrations to in vivo C_{max} or C_{ss} can generally be considered suitable to provide relevant and conservative OED estimates. Appendix B provides further details regarding the choice of the in vivo internal exposure metric for QIVIVE.

4.2 | In vitro exposure metrics for QIVIVE

No specific reference points for the test systems are recommended in this Scientific Opinion for the QIVIVE of DNT IVB data. Information on reference points considered relevant for the test systems of the DNT IVB is available in OECD (2023a). Besides performing QIVIVE based on effect concentrations, it may also be relevant to perform QIVIVE for NOECs for a better interpretation of negative results.

The PPR Panel acknowledges that understanding in vitro distribution kinetics is essential for robust QIVIVE as it provides insight into both the unbound concentration in the exposure medium and the cell-associated concentration. For an optimal linking of the in vitro (no) effect concentrations with the internal exposure metric in vivo used in PBK modelling, study of the in vitro distribution kinetics is highly recommended and considered important for addressing uncertainties in QIVIVE outcomes (see Section 7.1).

The PPR Panel also acknowledges that most available in vitro toxicity studies report only nominal concentrations with no or limited data on unbound concentrations in the medium or cell-associated concentrations. Few studies investigate how in vitro exposure metrics affect the outcome of QIVIVE, despite the OECD's GIVIMP document (OECD, 2018, annex H: Biokinetics & Xenobiotic Bioavailability) emphasising that nominal concentrations – the amount of chemical added to the exposure medium divided by the volume of exposure medium – may not reflect the biologically relevant dose because chemicals can bind to medium and cell constituents, such as serum proteins and cell lipids, microtiter plate plastics or they can evaporate. GIVIMP recommends focussing on unbound concentrations, which better represent what cells in vitro are actually exposed to.

Where only nominal effect concentrations are available, the PPR Panel considers it meaningful to perform QIVIVE based on nominal concentrations, provided that the necessary uncertainties are clearly identified, and it can be indicated whether this approach is expected to generate a conservative OED estimate. For non-volatile, stable chemicals with minimal transporter affinity, binding to plastic and sorption to cells and/or medium constituents, unbound concentrations are expected to be similar to nominal concentrations. For such chemicals, a high unbound plasma fraction is also expected in vivo and linking the nominal in vitro concentration to the total in vivo concentration would be close to linking the unbound in vitro and unbound in vivo concentration, thus providing a relevant starting point for the QIVIVE. Caution is warranted when chemicals are potent (e.g. with a median effect concentration, $EC_{50} < 100$ μM), volatile (e.g. with a log air/water partition coefficient, log $K_{AW} > -3$), unstable in solution (e.g. with a half-life of < 3 h) and/or lipophilic (e.g. with a log $P > 3$) (Birch et al., 2019; Groothuis et al., 2015; Proença et al., 2021; Riedl & Altenburger, 2007). It is noted that exceptions exist, e.g. certain chemicals with limited lipophilicity showing high protein binding (Henneberger et al., 2016). For more details with regards to the chemical and assay properties affecting the biologically relevant dose in vitro, see Appendix B.

If no experimental in vitro distribution kinetic study is available where unbound medium and cell-associated concentrations of the test chemical and/or relevant metabolites have been analytically determined over time in the DNT IVB assays, before using the nominal concentration as such, mass balance models may be used to quantitatively estimate how close the nominal concentration is to model estimates of unbound medium and cell-associated concentrations at steady-state. Several in silico steady-state mass balance models have been developed to estimate in vitro chemical disposition, which include the model described in Kramer (2010), Kramer et al. (2012), Armitage et al. (2014, updated in 2021; Armitage et al., 2021), Fischer et al. (2017) and Fisher et al. (2019). These models use readily available physicochemical properties such as molecular weight, acid association constant (pKa), octanol/water partition coefficient (log *P*) and Henry's law constant (*H*), as well as the assay-specific parameters, such as cell density, microtiter plate dimensions, exposure medium volume as well as protein and lipid content, to estimate unbound and cell-associated concentrations in in vitro systems. Although these models have not yet been applied to estimating the in vitro distribution kinetics of chemicals in the DNT IVB assays, they can offer a first indication of the conservativeness of nominal concentration-based QIVIVE by comparing OEDs derived from nominal concentrations with OEDs derived from model-estimated concentrations. Further evaluation of model applicability domains and prediction accuracy is still needed for these mass balance models (Proença et al., 2021; Scherer et al., 2024), including their applicability to the test systems of the DNT IVB. Uncertainties in model estimates of unbound and cell-associated concentrations should be noted, especially given that these models generally do not capture dynamic processes, such as evaporation and degradation (Proença et al., 2021; see Section 7).

As indicated before, derivation of in vitro AUCs can be complex, and in the absence of measured time-dependent cellular exposure and/or reliable in silico assessment of in vitro distribution, determination of in vitro AUC may not be feasible.

5 | PBK MODELLING FOR QIVIVE

PBK modelling is a tool typically applied to link external exposure to internal exposure. A PBK model consists of a set of mathematical equations that describe ADME processes of a chemical in an organism. PBK models incorporate physiological and anatomical parameters (e.g. cardiac output, tissue volumes and blood flows) along with chemical-specific parameters (e.g. absorption rate constants, plasma protein binding, tissue:plasma partition coefficients, metabolism and excretion rate constants). Together, these inputs allow for the simulation of a chemical's movement and biotransformation throughout the body following exposure via one or multiple routes.

An important step of the QIVIVE is the selection (or development) of an appropriate PBK model. This depends on several aspects, including the model complexity necessary and the availability of kinetic data for model parametrisation and evaluation.

This section provides information on the available guidance documents for PBK modelling (Section 5.1) and focuses on PBK modelling for QIVIVE of DNT IVB data for the hazard and risk assessment of pesticide active substances in the EU, considering species to be modelled (Section 5.2), different life stages to be modelled (Section 5.3), selection of PBK model for QIVIVE (Section 5.4), interpretation of QIVIVE outcome and consideration for high-tier PBK modelling (Section 5.5) modelling software and platforms (Section 5.6), model parameterisation (Section 5.7) and model evaluation (Section 5.8).

5.1 | Guidance documents on the development and use of PBK models

Several guidance documents on the development and use of PBK models have been published to support risk assessors and model developers in applying these models for chemical risk assessment and drug development. In Annex B, a tabular summary of these documents and their main features is provided.

In 2006 the U.S. EPA released a report to provide the first guidance on the use of physiologically based pharmacokinetic (PBPK) models in risk assessment (US EPA, 2006). Targeted primarily at U.S. EPA scientists and risk assessors unfamiliar with PBK modelling, it outlines the types of data and model requirements for evaluating a model's suitability for risk assessment. This was subsequently followed by an international effort from the WHO-IPCS, which published a guidance document in 2010 providing principles and best practices on the characterisation and application of PBK models in risk assessment (WHO, 2010). Then, in 2014, EFSA released a generic guidance document on good modelling practices that addressed various types of mathematical models (EFSA PPR Panel, 2014). This guidance focused rather on mechanistic effect models for the risk assessment of plant protection products and was not tailored to PBK modelling per se. During the same period, a drafting group at the European Committee for Standardization (ECS or CEN) developed a reporting framework for large chemical exposure models in a standardised format as part of the EU 4FUN project, which also developed the 'Merlin-Expo' software that incorporates PBK models.[15] This framework aimed to enhance the transparency and accessibility of large exposure models by defining documentation requirements and presentation standards (Altenpohl et al., 2018).

The US Food and Drug Administration (FDA) and European Medicine Agency (EMA) also released reporting guidelines to help standardise PBK model submissions in the pharmaceutical sector (EMA, 2018; FDA, 2018).

The European Scientific Committee for Consumer Safety (SCCS) considers all available scientific data, including PBK modelling, in the safety evaluation of cosmetic substances. Its most recent *Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation* adopted in 2023 (SCCS, 2023) defines the conditions for the use of PBK models submitted for risk assessment purposes, referencing the abovementioned WHO guidance (WHO, 2010).

PBK models have traditionally been calibrated and evaluated with reference to in vivo kinetic data using test species. As the toxicity testing paradigm is shifting towards alternative testing approaches, there is also an increasing need to develop PBK models based largely or entirely on in vitro and/or in silico data. To address this need, the OECD published a guidance in 2021 (OECD, 2021) on reporting criteria to evaluate PBK models developed using alternative approaches. The document includes a scientific workflow for characterising and validating PBK models with the emphasis on the use of in vitro and in silico data and an assessment framework for evaluating models with the emphasis on identifying major uncertainties underlying model inputs and outputs. To help end-users submit or evaluate a PBK model submitted for regulatory purposes, the guidance document also includes a template for documenting a model and a quality evaluation checklist. While the OECD guidance provides contextual information on the scientific process of PBK model characterisation and validation, it is not intended to provide technical guidance on PBK model development or best practices for modellers, which is addressed in other guidance documents, e.g. US EPA (2006), and WHO (2010).

The abovementioned PBK model guidance documents are broadly applicable to PBK models developed for humans, laboratory test species (e.g. rats, mice, dogs and rabbits), farm animals and ecologically relevant species (e.g. birds and fish). These guidance documents are applicable to PBK models for chemicals. In theory, the principles may be applied to chemicals in nanoform (nanomaterials), biologicals, macromolecules and metals but would need to be tailored to capture the additional features relating to the kinetics of these chemicals and the availability of in vitro and in silico methods to parameterise models for these substances.

[15] https://merlin-expo.eu/.

5.2 | Species considerations

The assays of the DNT IVB have been selected based on in vitro test systems available to cover the most important developmental processes related to neuronal development, for which chemical-induced disturbance is considered relevant for neurodevelopmental liabilities. Of the 17 assays/readouts, 4 of them are based on rat test systems and 13 on human test systems. Even though the in vitro toxicodynamic effects are captured in test systems for the different species, the PPR Panel does not recommend to solely perform QIVIVE with rat PBK models for rat test systems and with human PBK models for human test systems. Instead, conducting QIVIVE for all assays/readouts using both rat and human PBK models is recommended. This dual-species approach enables cross-species comparison, links in vitro findings to existing rat in vivo data (which is currently the basis for derivation of the reference point in the current EU regulatory practice) and provides human-relevant OED estimates. However, model choice should ultimately be made on a case-by-case basis, considering the assay species, mechanistic relevance of the endpoint, regulatory context and data availability. Overall, this strategy aligns with existing animal toxicity datasets (e.g. NOAELs/LOAELs), allows direct estimation of human-relevant doses and improves the robustness and regulatory relevance of QIVIVE outputs.

In general, in vivo kinetic data are more readily available for rats, which may be needed for PBK model parameterisation and is often considered necessary for model evaluation. Also, for pesticide active substances, ADME data from in vivo studies are typically available for rats. In the presence of a relevant and reliable in vivo kinetic dataset used for PBK model parameterisation and evaluation (valid PBK model), the resulting QIVIVE can be considered robust as it integrates in vitro effective concentrations with toxicokinetic modelling to derive biologically meaningful in vivo exposure metrics. As indicated in Section 5.5, QIVIVE-based OEDs can provide insight into whether available NOAELs/BMDLs for the active substance under evaluation are considered protective for effects obtained in the DNT IVB. When rat-based in vitro assays are used, rat PBK models allow comparison with NOAELs and LOAELs from rat studies available in the data package of the pesticide active substance under evaluation. For assays using human cells, human PBK models are more appropriate to extrapolate in vitro effect concentrations to human-relevant OEDs, but extrapolation with rat PBK models may also be of interest. A related in-depth assessment is recommended, e.g. evaluating whether there are indications of systemic or developmental toxicity at the level of rat OEDs in the available toxicity studies.

Inclusion of human PBK models in the QIVIVE provides insight into species differences in toxicokinetics and enables estimation of human OEDs. Such information supports the overall evaluation of whether the available TRVs for the active substance under evaluation[16] are considered sufficiently protective for effects obtained in the DNT IVB. Insights in human kinetics are not only relevant for the interpretation of in vitro toxicity data using a QIVIVE approach but also for assessing whether the standard uncertainty factors applied for TRV derivation sufficiently cover the interspecies differences in kinetics. If intraindividual differences are adequately described by the human PBK model, one may also evaluate whether human inter-individual differences in kinetics are sufficiently covered by the standard uncertainty factor. It must be noted, though, that for pesticide active substances, limited in vivo kinetic data will be available for human PBK model parameterisation and evaluation. Regulation (EC) No 1107/2009 indicates that '*For ethical reasons, the assessment of an active substance or a plant protection product should not be based on tests or studies involving the deliberate administration of the active substance or plant protection product to humans with the purpose of determining a human 'no observed effect level' of an active substance. Similarly, toxicological studies carried out on humans should not be used to lower the safety margins for active substances or plant protection products*'. Therefore, in vivo kinetic studies in humans are not recommended to be performed for PBK model parameterisation and evaluation for pesticide active substances in the EU. For PBK model validation, for which evaluation using in vivo kinetic data is considered the standard, other approaches as described in OECD (2021), such as read-across approaches, may be considered. If in vivo kinetic data have been reported, these are to be considered for the PBK model parameterisation/evaluation. For further considerations of PBK model parameterisation, see Section 5.7. In relation to the legal text quoted above, comparative toxicokinetic assessments using rat and human PBK models cannot be currently used to lower the standard uncertainty factors for the derivation of TRVs; however, model-related uncertainties and human-specific parameters may be considered in the weight of evidence for the overall risk characterisation, without altering the default UFs.

5.3 | Life stage considerations

OECD (2025) states: '*The assays within the DNT IVB are designed to evaluate chemical effects on key neurodevelopmental processes, such as proliferation and neurite outgrowth, which occur at various developmental windows and in different brain regions. The underlying hypothesis is that these processes are highly conserved, and as such, a chemical affecting one or more of these assays has the potential to elicit similar effects in vivo regardless of the specific timing or brain region involved.*' As this Scientific Opinion focuses on QIVIVE of data generated from the DNT IVB, the PBK modelling should capture internal exposures relevant to the development of the brain. This development spans from the developing embryonic/fetal stage through the juvenile period, during which chemicals may adversely affect neurodevelopment. When in vitro assays span

[16]TRVs are typically derived from a reference point (e.g. NOAEL or benchmark dose for the most sensitive endpoint, not necessarily related to DNT effects) from critical animal toxicity studies, applying uncertainty factors to account for inter- and intraspecies variability and possible data gaps. TRVs are interpreted as exposure levels below which adverse effects are not expected in humans. TRVs are agreed during the peer review process of pesticide active substance in the EU.

multiple stages or when the relevant life stage cannot be unambiguously assigned, PBK modelling should consider all applicable stages of neurodevelopment, considering that internal exposure in different life stages may differ with same external exposure.

In that respect, the following life stages (in line with OECD 2025) with related exposure scenarios are relevant for QIVIVE of DNT IVB data:

1. Developing embryo/fetus: internal exposure occurs in the developing embryo and fetus; external exposure to the pesticide active substance is via the mother.
2. Breastfed infant: internal exposure is to the infant via breast milk exposure; external exposure to the pesticide active substance, relevant for the derivation of the OED, is via the lactating mother.
3. Not-breastfed infant: internal exposure is to the not-breastfed infant; external exposure to the pesticide active substance is directly to the not-breastfed infant (age-appropriate dietary sources).

5.4 | PBK model selection

As indicated in OECD (2025), parsimony should guide the selection of a model structure, meaning that it should include only the essential parameters needed to adequately represent the physiology of the target species, the key ADME characteristics of the chemical and relevant exposure scenarios. A model should be as complex as necessary to achieve its intended purpose without introducing unnecessary complexity. In the absence of reliable data or robust biological basis for parameterisation or evaluation, a PBK model may produce unreliable predictions and diminish its utility (Kilkenny & Robinson, 2018). It may also create a false sense of confidence, implying greater predictive power or precision than what it can deliver. Therefore, the OECD QIVIVE document (OECD, 2025) proposes using a tiered approach for selecting a PBK model structure, guided by the availability of data for model parameterisation and evaluation, the intended purpose and level of uncertainty acceptable for the context of use.

The following tiers are described in the OECD QIVIVE document (OECD, 2025):

- Tier 0: High throughput adult or juvenile models to predict maternal plasma or juvenile plasma concentrations.
- Tier 1: Whole-body adult or juvenile models to predict maternal plasma or juvenile plasma concentrations. The complexity can be increased by providing accurate description of (local) brain concentrations, which can be considered for the juvenile models (Tier 1+).
- Tier 2:

  a. Gestational models to predict fetal plasma and/or brain concentrations.
  b. Lactational-infant models to predict plasma and/or brain concentrations in nursing infants.

The PPR Panel does not recommend Tier 0 models for the hazard and risk assessment of pesticide active substances in the EU as these models are more adequate for screening/prioritisation approaches and since they lack tissue compartments to estimate tissue (including brain) exposure. Therefore, it is suggested to begin with Tier 1 for PBK modelling applied for the QIVIVE of DNT IVB data for the hazard and risk assessment of pesticide active substances in the EU. Tier 2 models may provide higher precision of OEDs, but if not sufficiently supported by data, the uncertainty in such PBK model outcomes and related OEDs increases. Therefore, if insufficient data are available to parameterise/evaluate such high tiered PBK model, their use is not recommended.

Given that there is the general absence of in vivo kinetic data on fetal (internal) exposure for most pesticide active substances for model parameterisation and evaluation, Tier 1 PBK models should be used as the first step in QIVIVE, provided that they yield sufficiently conservative internal exposure estimates. Metrics to be considered (and described by the PBK model) include plasma concentration (total and unbound) and brain concentration (see Section 4 for details on exposure metric selection). For early phase pregnancy (first trimester), one could consider linking cell-associated concentrations to uterus concentrations, assuming that the concentration in the uterus resembles that of the implanted embryo. In Appendix C, detailed considerations have been provided to support that Tier 1 PBK model-based QIVIVE generally provides relevant and conservative OEDs for pregnant woman and non-breastfed juveniles for chemicals that do not bioaccumulate. Kinetic data selected to support that analysis is presented in Annex C.

The OECD QIVIVE document (OECD, 2025) considers a whole-body adult or juvenile model for Tier 1 modelling. Regarding the adult model, this may be tailored to pregnant women, i.e. applying physiological parameter values for pregnant women to the Tier 1 models (without the need for description of a fetal compartment as this would be a Tier 2 model). Such models would cover life stage of the developing embryo/fetus and its indirect exposure to pesticide active substance via the mother, considering maternal plasma concentration as a conservative internal exposure metric for the QIVIVE (see Annex C for detailed considerations).

Juvenile PBK modelling can be considered specifically if there is evidence that juvenile biotransformation differs significantly from adults, potentially resulting in higher internal exposure (e.g. as reported for deltamethrin, Kim et al., 2010; cypermethrin, Liu et al., 2019). Such juvenile models would cover the life stage of a not-breastfed infant and its direct exposure to the pesticide active substance.

It is acknowledged that Tier 1 models for pregnant women and infants do not cover the life stage of breastfed infants and its indirect exposure to pesticide active substance via breast milk. As also indicated in the OECD QIVIVE document (OECD, 2025), infant exposure via breast milk (i.e. from maternal exposure) is not described by Tier 1 PBK models. The PPR Panel considers that estimation of the internal exposure of the infant via breast milk is complex particularly due to the lack of kinetic data needed to develop/evaluate PBK models to obtain OEDs for lactating dams/mothers, also considering uncertainties related to infant dosing, being a function of milk concentration, feeding patterns and infant ADME.

An evaluation was conducted to determine if any kinetic data are available for pesticide active substances that have harmonised classification under hazard statement H362 ('may cause harm to breastfed children'), i.e. for pesticide active substances for which information on exposure via breastmilk is deemed relevant. The pesticide active substances (beta-) cyfluthrin, etofenprox, fenarimol, flufenoxuron, fluxapyroxad, lindane, metaflumizone, mirex, trifloxystrobin and triadimenol have harmonised classification under hazard statement H362. Related information on these pesticide active substances is summarised in Annex D. In particular, Annex D entails data on the abovementioned H362-classified substances, including the rationale for this classification, availability of kinetic data and their potential transfer into milk (in rodent, goat and livestock). The resulting data table highlights the heterogeneity of available data; for certain substances such as etofenprox and flufenoxuron, rodent and livestock (goat/cow) studies provide information on the quantity of the substance and/or its metabolites in milk as well as ADME characteristics. In contrast, for other substances like beta-cyfluthrin and triadimenol, data on milk transfer is lacking or incomplete. The H362 classification relied on observed adverse effects in pups, such as tremors (beta-cyfluthrin) or reduced viability and growth (triadimenol), suggesting transfer of chemical via milk without direct quantitation to support exposure via milk.

The available data, particularly from livestock metabolism studies with radiolabelled substances, can be used to identify major components of the terminal residue in edible tissues (residue in edible tissues relevant for consumer risk assessment), including milk. These studies aim to establish at least 90% of total radioactive residue (TTR) in milk and determine if a residue is fat-soluble and thus its potential to accumulate in milk. Such information could be relevant as input parameters in PBK modelling to predict breast milk transfer. However, experimental data on quantification of pesticide residues in milk are generally lacking for most pesticides; this is not only true for rodents but also for goats and ruminants. Overall, the rationales described for the H362 classification are linked to findings during the lactational period but are not linked to DNT endpoints, and related kinetic information is generally insufficient to parameterise PBK models for lactational transfer.

Since the relevance of exposure of breastfed infants cannot be excluded, it should be justified whether the QIVIVE outcomes for other life stages would provide a sufficiently conservative OED that is similar to or lower than that expected for the lactating mother (see Figure 4). If so, PBK modelling of the internal exposure for the lactating mother and/or breastfed infant would not be needed. Notably, an OED based on pregnant women may be overconservative for lactating mothers, e.g. if this transfer is very low or absent, the OED based on pregnant woman may overestimate internal exposure for the breastfeeding scenario.

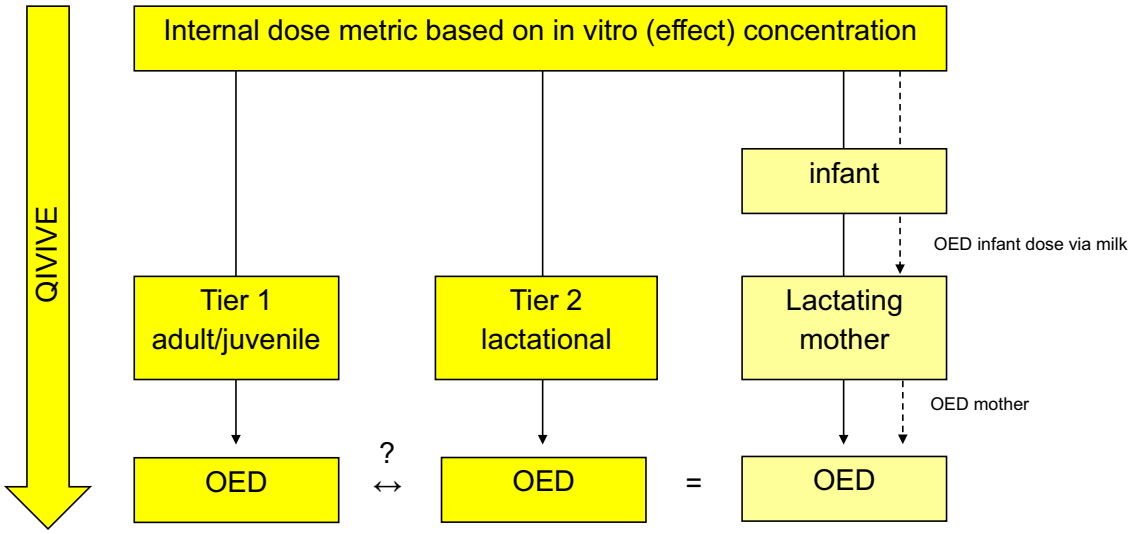

FIGURE 4 Schematic overview of reverse dosimetry-based QIVIVE based on Tier 1 model and for higher tier models describing breastfed infants. It should be justified whether the OED predicted from Tier 1 models is expected to cover the OED for lactating mothers. For the lactating mothers, it is schematically depicted that the OED for the mother is based on the OED of the breastfed infant.

An assessment on lactational transfer of chemicals may be cumbersome without specific kinetic data on this. Chemicals with limited maternal clearance and high milk transfer are more likely to result in relatively high breast milk concentrations and, consequently, high external infant exposure (Ito, 2000; Verstegen et al., 2022). Thus, recommended aspects to consider include information on maternal and infant clearance as well as plasma to milk transfer. Chemical characteristics that may play a role include plasma protein binding, milk binding, ionisation grade and being a substrate of specific transporter proteins. Special attention should be paid if the capacity of the infant to clear or excrete the chemical is limited. Reduced

clearance has been reported to play a role in adverse effects in breastfed infants from mothers exposed to drugs (e.g. atenolol, carbetapentane, dapsone, doxepin, fluoxetine and lithium) as summarised in Verstegen et al. (2022). For pesticide active substances, reduced metabolic clearance in juveniles has been reported in rats for deltamethrin (Kim et al., 2010) and cypermethrin (Liu et al., 2019), indicating potential for higher internal exposure in early life stages.

The PPR Panel recommends collecting information to obtain insight into the chemical's maternal clearance, transfer to milk and infant clearance. Suggestions for that are provided below:

- Regarding information on maternal clearance, in vivo kinetic data in rats generated from required ADME studies for pesticide active substances can provide some information on this (see Annex E for more information on regulatory ADME studies). In vitro comparative metabolism studies, also a data requirement for pesticide active substances in the EU and if properly conducted, may provide relevant insight into and comparison of metabolic clearance in rats and humans. An estimation of maternal clearance can be made with an adequate Tier 1 PBK model.
- Regarding transfer to milk, experimental data to estimate infant exposure (in relation to maternal external exposure) are generally not available for pesticide active substances. However, Abduljalil, Pansari, et al. (2021) and Pansari et al. (2022) used a PBK modelling approach to predict milk-to-plasma ratios for various pharmaceuticals based on models by Fleishaker et al. (1987) and Atkinson and Begg (1990) that used the fraction unbound in maternal plasma and milk. In the absence of experimental data to determine the fraction unbound in milk, they may be estimated as demonstrated in Abduljalil, Pansari, et al. (2021). Zhang et al. (2022) also provided a perspective on the related equations by Atkinson and Begg (1990) and provided an R/Shiny interface for the model aiming to support scientists to acquire in-depth understanding of the theoretical background and implementation of the model. These estimations do not account for active transport. Transporter proteins, such as breast cancer resistance protein (BCRP), have been shown to play a role in milk secretion (Garcia-Lino et al., 2020). Therefore, information on whether a chemical is a substrate for such transporters can help assess the likelihood of milk transfer and subsequently assist in the evaluation of determining whether Tier 1 QIVIVE OEDs are sufficiently conservative.
- Regarding infant clearance, specific kinetic data on infant clearance for pesticide active substances are typically lacking. Pyrethroids such as deltamethrin (Kim et al., 2010) and cypermethrin (Liu et al., 2019) have been shown to have reduced metabolic clearance in juvenile rats in vivo. Such metabolic differences may also be relevant for humans as the main enzymes responsible for metabolic clearance of these parent chemicals (carboxylesterases) have a relatively low expression in the first 3 weeks after birth (Hines et al., 2016). However, limited infant clearance does not necessarily mean that a Tier 1 model would not be sufficiently conservative as levels that reach milk may also be relatively low, e.g. due to quick maternal clearance. Information on the main enzymes responsible for metabolic clearance, including isoform-specific metabolism information when specific biotransformation enzymes are involved, as well as knowledge on the ontogeny (life-stage specific expression/activity) can be helpful to evaluate possible life-stage dependent differences in metabolic clearance.

5.5 | Interpretation of QIVIVE outcomes and considerations for moving to high-tier modelling

Both reverse dosimetry and forward dosimetry QIVIVE approaches are considered relevant by the PPR Panel for the quantitative interpretation of the DNT IVB data. Reverse dosimetry may be particularly efficient when the OED from one assay is to be compared to various in vivo reference points, whereas forward dosimetry may be more efficient when multiple in vitro reference points are compared to one internal concentration that corresponds to a single exposure scenario of interest. In the following, a proposed way of presenting the outcomes using forward and reverse dosimetry is provided and considerations are provided when moving to high-tier modelling can be considered based on the outcomes of Tier 1 PBK model-based QIVIVE.

5.5.1 | Reverse dosimetry-based QIVIVE

As an outcome of a reverse dosimetry-based QIVIVE analysis, the OEDs can be compared with the reference points (such as NOAEL/BMDL values) available in the data package considered for TRV derivation of a pesticide active substance. Main possible scenarios and interpretation are described below and are schematically presented in Figure 5.

- If the OEDs are much higher than the reference point and TRV, one may conclude that the available TRV is considered sufficiently protective (Figure 5A). In other words: even at or above the TRV, internal concentrations are expected to remain below levels that cause effects in the DNT IVB assays. Currently, no value for a sufficient margin between OEDs and available reference points can be defined. This is to be decided on a case-by-case basis and may depend on the in vitro reference point selected and other evidence available.
- If the OEDs are much lower than the reference point and TRV, one may conclude that the available TRV is not considered sufficiently protective (Figure 5B). If the OED is based on a Tier 1 model and the internal exposure is potentially overconservative, higher tier modelling can be considered (for which kinetic data generation may be needed) and the resulting

OEDs should be interpreted. If the Tier 1 model-based OED is the final outcome of the QIVIVE (or if the OED is based on a Tier 2 model), the uncertainties should be considered for the risk assessment and the application of an extra uncertainty factor for TRV derivation or the application of an increased margin of safety might be included. Also, other follow-up actions could be considered to assess the DNT hazard. Currently, the size of the margin between OEDs and available reference points cannot be defined what is considered 'much lower'. This is to be decided on a case-by-case basis and may depend on the in vitro reference point selected and other evidence available. However, this scenario considers that possible refinement of the low-tier PBK modelling approach (such as by new studies to reduce uncertainty in PBK model parameters and/or to reduce uncertainty in the in vitro exposure metric) would not be sufficient to change the conclusion on the protectiveness of the TRV.

- If the OEDs are close to the reference point and TRV, one may conclude that the available TRV may also not be sufficiently protective (Figure 5C). In such cases, it could be considered whether the QIVIVE approach can be refined, e.g. by performing studies to improve PBK model parameter estimation and/or by performing in vitro distribution kinetic studies to provide better insight in the in vitro exposure metric used in the QIVIVE. Also, if the OED is based on a Tier 1 model and the internal exposure may be overconservative, higher tier modelling can be considered (for which kinetic data generation may be needed) and the resulting OEDs should be interpreted. If the Tier 1 model-based OED is the final outcome of the QIVIVE (or if the OED is based on a Tier 2 model), the uncertainties should be considered for the risk assessment and application of an extra uncertainty factor for TRV derivation or the application of an increased margin of safety in the risk assessment might be included. Also, other follow-up actions could be considered to assess the DNT hazard.

For the PBK modelling for QIVIVE of DNT IVB data for the hazard and risk assessment of pesticide active substances in the EU, the PPR Panel recommends always starting with Tier 1 modelling and applying higher tier modelling if triggered and/or where sufficient data for related model parameterisation and evaluation are available.

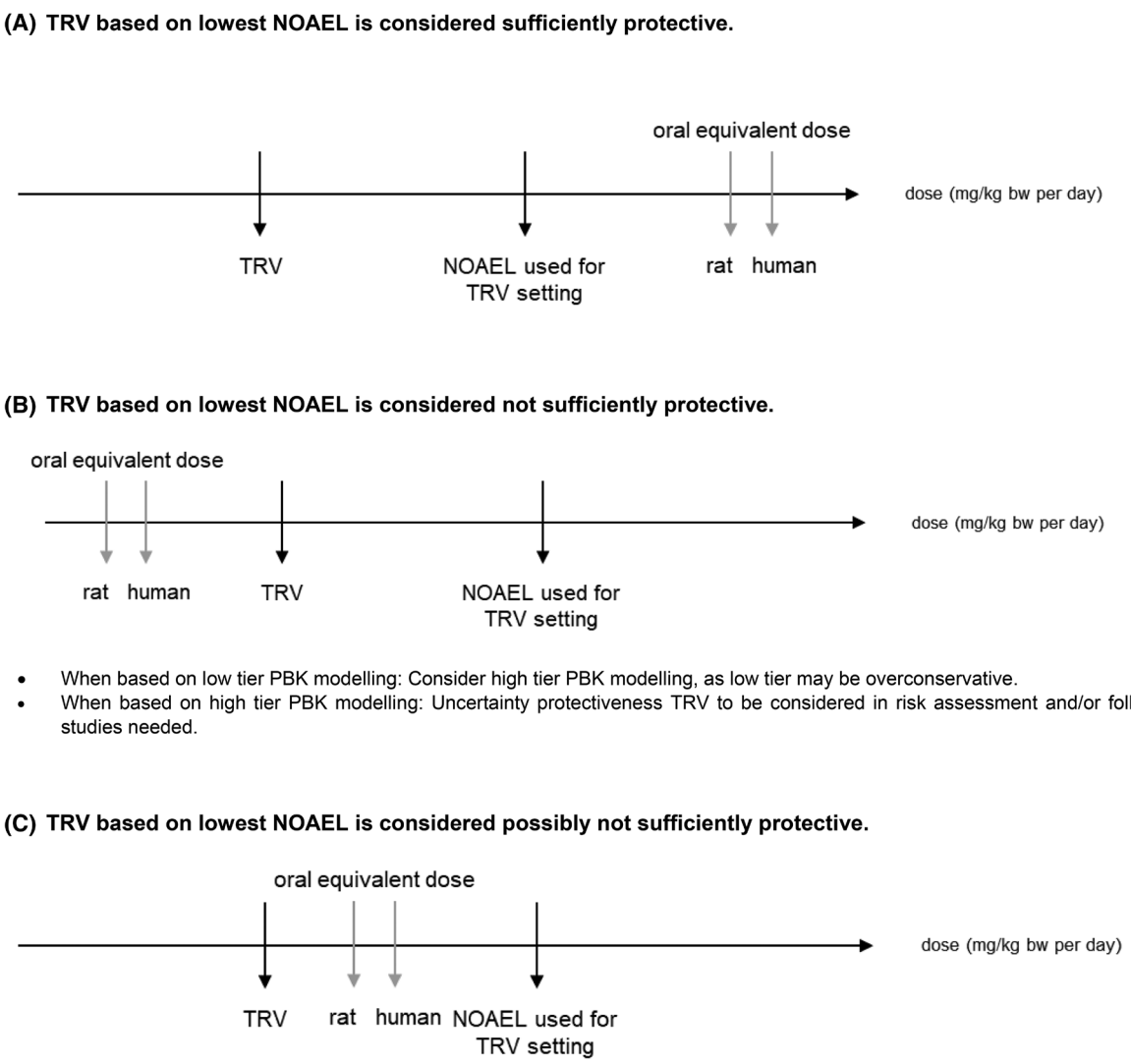

FIGURE 5 General scenarios for interpretating QIVIVE outcomes using a reverse dosimetry approach: Comparing the OEDs to available reference points (NOAELs used for TRV setting) in the data package for the pesticide active substance and the related TRVs. See text in this section for more details on interpretation. For readability of the figure, the OEDs are shown as point estimates. Ideally, information on variation in PBK model parameters and/or quantified uncertainty in model parameters is included in the model outcome (providing a distribution of values for the OED). Only one TRV has been presented, whereas one can consider different TRVs. Likewise, only reference points (NOAELs used for TRV setting) are presented, but consideration of related LOAELs and or NOAELs/LOAELs from other studies can also be useful for the interpretation of QIVIVE outcomes. It is also noted that in this example, the rat OED is slightly lower than the human OED, but OEDs may largely differ (i.e. with major species differences in toxicokinetics) and/or human OEDs may be lower than rat OEDs.

5.5.2 | Forward dosimetry-based QIVIVE

When applying a forward dosimetry-based QIVIVE, a logical starting point in the context of this Scientific Opinion is an available TRV based on the dataset available for the pesticide active substance. One advantage of a forward dosimetry approach compared to a reverse dosimetry approach is that it allows to visualise separately the quantification of the uncertainty/variation in the PBK modelling-based estimation of the internal exposure versus the uncertainty of the in vitro exposure metric used for the QIVIVE. A possible way of presenting the related outcome of such an approach is shown in Figure 6.

In the example displayed in Figure 6, the PBK modelling-based predicted internal exposure estimate is presented applying a daily exposure to a TRV, e.g. the ADI. These predicted internal concentrations are ideally presented as a range, i.e. considering variation in the population modelled as well as uncertainty in model parameters. The in vitro exposure metrics relevant for the QIVIVE (see Section 4) are also shown as ranges, representing uncertainty. If available, insight into the shape of the distribution (i.e. bell-shaped or left-or right-skewed) is helpful for assessing whether the TRV is considered sufficiently protective related to the findings of these in vitro assays. In Figure 6A, a conclusion could be that the TRV is possibly not considered sufficiently protective regarding the findings in assay 1. A targeted approach may be performed to refine the assessment. If PBK modelling-derived distribution only covers biological variation, this cannot be reduced. If

it also covers uncertainty in model outcome related to uncertainty in parameter values, it may be evaluated whether experiments can be performed/evidence can be collected to reduce this uncertainty. In Figure 6B, uncertainty in the in vitro exposure metric was reduced by performing an in vitro distribution kinetic study.

Considerations regarding moving from a Tier 1 modelling approach to a higher tier modelling approach are the same as for applying a reverse dosimetry-based QIVIVE as described in Section 5.5.1.

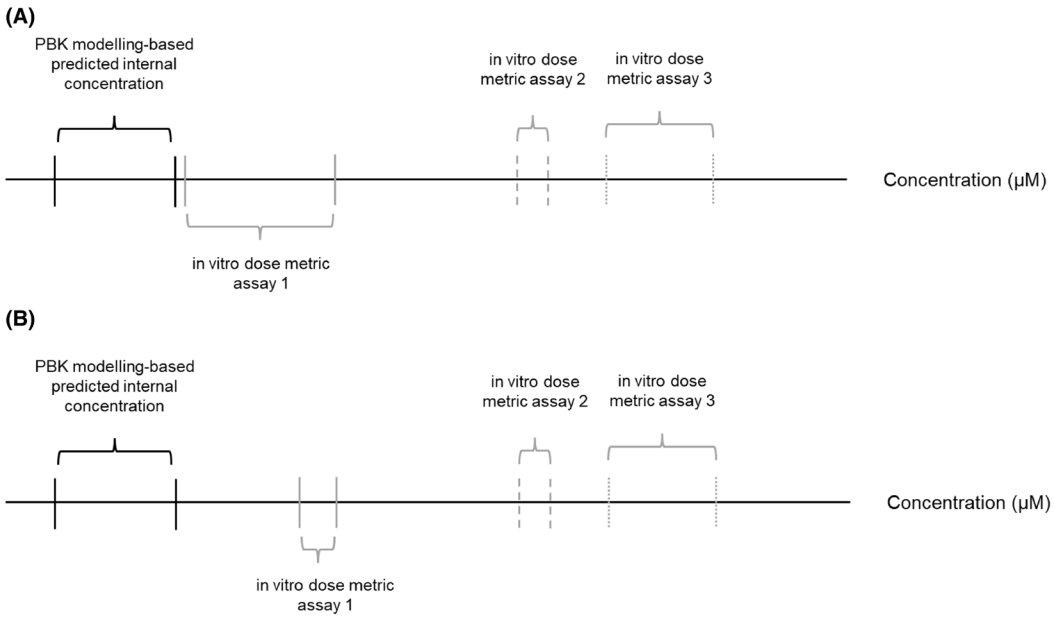

FIGURE 6 Hypothetical outcome of the QIVIVE assessment using a forward dosimetry approach for a chemical for which 3 of the DNT IVB assays showed a response in vitro, comparing the PBK modelling-predicted internal concentration at the acceptable daily intake (ADI) to the in vitro exposure metrics considered relevant for the QIVIVE of DNT IVB assays with a positive response. In this example the uncertainty in the in vitro exposure metric for assay 1 was reduced (B vs. A) by performing an in vitro distribution kinetic study. For clarity of the Figure, one PBK modelling-predicted internal concentration is presented here, but more are to be considered (e.g. for rat and human).

5.5.3 | QIVIVE based on high-tier PBK modelling

Certain outcomes of QIVIVE based on low-tier PBK modelling may trigger the use of higher tier PBK modelling (see for more considerations Section 5.5.1). Also, if sufficient kinetic data are available for higher tier PBK model parameterisation, their inclusion is recommended when related OEDs are considered more accurate than those obtained with Tier 1 PBK modelling.

In the absence of adequate kinetic data to develop and/or evaluate such high-tier PBK models, the related outcomes of QIVIVE can be considered as highly uncertain and one should refrain from using such high-tier modelling as also indicated in the OECD QIVIVE document (OECD, 2025).

The PPR Panel notes that various higher tier PBK models have been described in the literature and that reported models have been listed in an appendix of the OECD QIVIVE document (OECD, 2025). It is important to note that inclusion in that list does not imply endorsement, validation or regulatory acceptance of the PBK models or the overall approach presented in the studies by the OECD or other regulatory agencies. Four published QIVIVE case studies for the quantitative interpretation of in vitro DNT data (not necessarily data from the DNT IVB) have been reported in the OECD QIVIVE document (Algharably et al., 2023; Dobreniecki et al., 2022; Johansson et al., 2024; Maass et al., 2023). Among these studies, three of them used complex PBK modelling (all pregnancy models), which would align to Tier 2 models as described in OECD (2025), i.e. describing fetal plasma and brain concentrations. However, in all three cases, very limited to no kinetic data were available for parameterisation and/or evaluation of fetal exposure, and as such, the predictions of fetal plasma and/or brain concentrations from these studies may be highly uncertain. Therefore, for not increasing uncertainty, even though codes for higher tier models (following the tiers as described in OECD, 2025) are available, they should only be applied if robust data support them, increasing the informative potential by their use.

5.6 | PBK modelling software and platforms

PBK modelling can be performed using various dedicated software tools or programming packages available in R and Python. These tools primarily function by solving systems of ordinary differential equations to simulate the ADME properties of a chemical in biological systems. A comprehensive list of commonly used PBK modelling software is provided in the OECD Guidance No. 331 (Annex 1 of OECD, 2021). Additionally, an inventory of PBK models designed to assess internal

exposure to contaminants across different life stages, accounting for age-dependent variations in organ physiology, metabolism and clearance from in utero to late life, is available in Ratier et al. (2023).

The PPR Panel does not endorse any specific PBK model or software for the QIVIVE of DNT IVB data. However, it is crucial that the selected software supports forward and reverse dosimetry, allowing estimations using either unbound concentration in plasma or total tissue concentrations (e.g. in the brain) depending on the QIVIVE scenario applied (see Section 4).

Both commercial and open-access software, including HTTK,[17] GastroPlus,[18] Simcyp Simulator,[19] PK-Sim,[20] PLETHEM[21] and TKPlate[22] (Bossier et al., 2023), are suitable for this purpose.

5.7 | PBK model parameterisation

Parameters required for PBK models can be divided into two main groups, one being the parameters describing the human or animal physiology (see Section 5.7.1) and the other being the parameters describing the chemical-specific ADME processes (see Section 5.7.2).

5.7.1 | Physiological PBK model parameters

Physiological parameters in PBK models are needed to describe the physiology of the animals or humans of interest, and do not depend on the chemical of interest. Such parameters have been gathered in review papers and databases and are sometimes included in PBK modelling platforms.

Certain models apply physiological information about an average animal/human, which may provide relevant low-tier estimates of internal exposure. Information about variation in the physiological parameters within a given population can be added in the model, which may either be based on assumptions and/or available data. Information about specific or more susceptible groups of animals/humans, such as physiological parameters for young children and pregnant women (Abduljalil, Pan, et al., 2021; Beaudouin et al., 2010; Dallmann et al., 2017), can also be applied when relevant, allowing the PBK models to cater for specific groups of interest.

The PPR Panel does not recommend specific physiological parameters to be applied in the PBK modelling for QIVIVE of DNT IVB data but considers it essential to have clear descriptions behind the selection along with the underlying data of the parameter values.

5.7.2 | Chemical-specific PBK model parameters

Besides the abovementioned physiological parameters, each PBK model also requires chemical-specific parameter values for the ADME processes it describes. There is no general harmonised approach to derive such parameters. Therefore, the PPR Panel considers it essential that, for PBK models intended for regulatory application, the parameterisation process is clearly described and the underlying data are thoroughly and transparently evaluated (see Section 8).

Regarding the use of PBK models for QIVIVE of DNT IVB data, the PPR Panel considers the chemical-specific parameter values as schematically presented in Figure 7 and further described below of particular interest for the ADME processes. Most of these parameters are applicable to both low- and high-tier PBK models, whereas some are only applicable to higher tier models.

[17] https://cran.r-project.org/web/packages/httk/index.html
[18] https://www.simulations-plus.com/software/gastroplus/.
[19] https://www.certara.com/software/simcyp-pbpk/.
[20] https://www.open-systems-pharmacology.org.
[21] https://scitovation.com/plethem/.
[22] https://r4eu.efsa.europa.eu/.

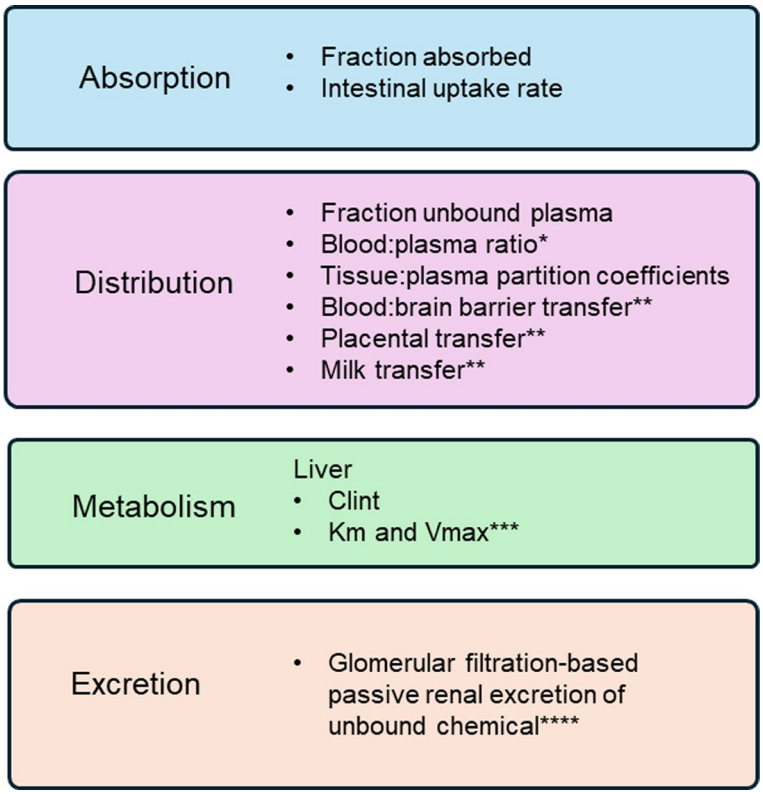

FIGURE 7 Recommended minimal ADME parameters for PBK models for QIVIVE. *Needed in case triggered by regulatory ADME study, **only needed for high-tier models, ***needed if nonlinear kinetics, e.g. saturation in biotransformation occurs, ****in case of no/limited biotransformation, more information on renal clearance and/or biliary clearance may be needed.

Kinetic information to support the parameterisation can be obtained using in vivo, in vitro and/or in silico methods. General considerations for deriving PBK model parameter values of pesticide active substances are described in Section 5.7.3. In addition, detailed information on a selection of in vivo, in vitro and/or in silico methods for chemical-specific parameterisation of PBK models is provided in Annex E.

5.7.2.1 | *Absorption*

- **Fraction absorbed (needed for both low- and high-tier models)**

To describe the oral absorption in the PBK model, information is needed on the fraction absorbed. The higher the fraction absorbed, the more of the chemical is available for internal exposure. Typically, in low-tier PBK models, description of the fraction absorbed in PBK models is not dependent on the dose, but if considered relevant, it can be described in a dose-dependent manner.

- **Intestinal uptake rate (needed for both low- and high-tier models)**

Information is also needed to parameterise the rate of chemical uptake from the GI-tract to the liver (via the portal vein) in the PBK model. The higher the uptake rate, the higher the C_{max} and lower the T_{max} (time to reach C_{max}).

5.7.2.2 | *Distribution*

- **Fraction unbound in plasma (needed for both low- and high-tier models)**

Chemicals may bind to plasma proteins, which has an important impact on their toxicokinetics. Binding to plasma proteins is typically described as the fraction of the concentration in plasma that is not bound to plasma proteins (the fraction unbound), which is often considered concentration independent. Fraction unbound in plasma is required as input information for various calculations of tissue:plasma partition coefficients and to describe the passive renal excretion of chemicals.

As indicated in Section 4, the PPR Panel recommends performing QIVIVE and subsequently linking the unbound concentration in the culture medium in vitro to the unbound concentration in maternal plasma. The latter is derived from the total concentration in plasma multiplied by the fraction unbound in plasma.

- **Blood:plasma (B:P) ratio (needed for certain chemicals for both low- and high-tier models)**

Some chemicals bind extensively to red blood cells. In such cases, this needs to be considered in the PBK modelling by describing a so-called blood:plasma (B:P) ratio, which is defined as the blood concentration of the chemical divided by its plasma concentration. Regulatory ADME studies provide information on binding to red blood cells and may trigger the need for description of chemical partitioning into red blood cells in the PBK model.

- **Tissue:plasma partition coefficient (needed for both low- and high-tier models)**

This coefficient describes the partitioning of the chemical between a given tissue and the plasma. The higher the coefficient, the higher the tissue concentration in relation to the plasma concentration.

- **Blood:brain barrier (BBB) transfer (needed only if brain exposure needs to be refined in the higher tier modelling)**

Prediction of brain concentrations based on brain:plasma partition coefficient generally overpredicts the concentration of chemicals in the brain for which exposure is limited due to limited translocation across the BBB. Description of brain exposure may be refined through consideration of the BBB function (using available in vivo, in vitro or in silico data, at the modeller's discretion).

- **Placental transfer (needed only if fetal exposure needs to be refined in the higher tier modelling)**

In low-tier PBK modelling, unbound maternal plasma concentrations are considered to be adeqaute surrogates for unbound fetal plasma concentrations (see for considerations Appendix C) and when higher tier PBK modelling is needed to refine the description of fetal exposure, transfer of the chemical across the placenta needs to be considered.

- **Milk transfer (lactation; needed only in specific cases in the higher tier modelling)**

Description of milk transfer would be needed when modelling breastfed infants (see Section 5.3).

5.7.2.3 | *Metabolism*

- **Metabolic biotransformation (needed for both low- and high-tier models)**

Most pesticide active substances are extensively biotransformed, mainly in the liver, but also in other organs or blood as well as by the microbiota. For metabolism, nonlinear kinetics, e.g. saturation of biotransformation, is often considered in PBK modelling, but simple PBK models may describe biotransformation as a non-saturable process (e.g. by describing intrinsic hepatic clearance). Parameters for metabolic biotransformation (such as the metabolic clearance) are essential for PBK models.

5.7.2.4 | *Excretion*

- **Renal excretion (needed for both low- and high-tier models)**

Renal clearance is important to describe the urinary excretion of pesticide active substance and its metabolites. In case of extensive hepatic biotransformation of the parent chemical, renal excretion is not considered to play an important role in the excretion of the parent chemical but rather more for the excretion of the metabolites. However, in cases of limited metabolic biotransformation, renal clearance may significantly influence the internal concentrations of the parent pesticide active substance and contribution of transporter-mediated excretion and/or reabsorption may need to be described in the PBK model.

- **Biliary excretion (needed for certain chemicals for both low- and high-tier models)**

Biliary excretion is an important excretion route for various pesticide active substances in rats, in many cases related to excretion of metabolites. As for renal excretion, in case of extensive hepatic biotransformation of the parent chemical, biliary excretion has a limited role in the excretion of the parent chemical, but more for the excretion of the metabolites. In those cases, biliary excretion is not expected to largely affect the internal concentration of the parent chemical.

In case there is direct biliary excretion of non-biotransformed parent chemical, description of biliary excretion in the PBK model is to be considered for both low- and high-tier models.

5.7.3 | Data sources and methods for chemical-specific model parameterisation

Since very few test guidelines are available for methods used for PBK model parameterisation, the PPR Panel considers it essential to have a critical assessment of study setup and data analysis of the studies used to generate data for PBK model parameterisation.

In this section, some general considerations are provided while more detailed information is presented in Annex E, also considering the kinetic data typically available in the pesticide active substance dossiers in the EU.

This Scientific Opinion focuses on the chemical-specific parameter values of the parent chemical, allowing QIVIVE of in vitro DNT IVB data of the parent chemical. If QIVIVE is to be performed for one or more metabolites, kinetic data on metabolites(s) are also needed to allow an adequate description of the metabolite(s) of interest.

5.7.3.1 | *General considerations on in vivo kinetic studies in animals*

In dossiers of pesticide active substances submitted under the framework of Regulation (EC) No 1107/2009, which regulates the approval of active substances and products within the EU, comprehensive ADME data are indispensable for the hazard and risk assessment of these substances. The data requirements on ADME properties of the active substance are laid down in Commission Regulation (EU) 283/2013. ADME data serves several critical purposes, including providing insight into internal exposure metrics, allowing to correlate internal exposure levels to toxicological outcomes. They also provide information on the accumulation potential of an active substance and play a key role in determining appropriate dose levels for toxicity studies. Lastly, these studies can identify potential biomarkers of exposure that can be used in human biomonitoring studies. Adherence to specific regulatory guidelines is necessary in conducting these studies. The OECD Test Guideline 417 (OECD, 2010) outlines procedures for toxicokinetic studies that assess the ADME of chemicals in laboratory animals.

In vivo ADME studies, conducted according to Commission Regulation (EU) 283/2013 and OECD Test Guideline 417 (OECD, 2010), provide essential information on how a substance is absorbed, distributed, metabolised and excreted in laboratory animals, typically rats.

- Absorption studies typically involve oral administration of a radiolabelled chemical and blood sampling at various time points to determine how much of the substance enters systemic circulation. Samples are often taken at intervals up to 24 h post-dose, using scintillation counting to detect radioactivity. Studies are generally performed in both sexes.
- Distribution studies use radiolabelled tracers to track the substance across tissues, highlighting potential accumulation in target organs. They can provide tissue-to-blood ratios and identify retention in organs such as liver, kidney, fat and brain, which may indicate potential for bioaccumulation.
- Metabolism studies identify the chemical transformations of the substance and its breakdown products, using analytical techniques such as LC–MS. They aim to characterise metabolic pathways and quantify parent chemical and metabolites in excreta.
- Excretion studies focus on how and to what extent the chemical and its metabolites are eliminated from the body, usually via urine and faeces. Animals are housed in metabolism cages to enable accurate recovery of excreta and determination of cumulative elimination.

The resulting toxicokinetic parameters – including fraction absorbed, C_{max}, AUC, T_{max}, half-life and clearance (CL) – are important for assessing internal dose and informing reference values for non-dietary exposure scenarios. However, these parameters are usually based on total radioactivity and often do not distinguish between parent chemical and its metabolites. This significantly limits the value of such data for PBK model parameterisation and evaluation. CL is usually estimated as dose/AUC based on total radioactivity, but organ-specific clearance (e.g. renal or hepatic) is rarely derivable from regulatory ADME data due to lack of specific concentration measurements.

The PPR Panel notes that in many cases, these studies provide limited data that are of use for PBK model parameterisation and evaluation as information on internal exposure (blood, plasma, tissues) is typically only available for total radioactivity (i.e. parent chemical and its metabolite(s)) and not for the parent chemical and its metabolite(s) separately. To make data from such regulatory ADME studies more relevant for PBK model parameterisation and evaluation, the PPR Panel highly recommends that, besides quantification of total radioactivity, the parent chemical and metabolite(s) are also quantified separately for the blood, plasma and tissue samples (see Section 10 on Recommendations). It is highlighted that if such analyses would be included in the regulatory ADME studies, adequate data for PBK model parameterisation and evaluation would be obtained without the need for additional in vivo studies for PBK modelling for QIVIVE.

In vivo kinetic data are often used for the following two purposes: (1) derivation of chemical-specific PBK model parameter values (model parameterisation) and (2) PBK model evaluation. In the former case, the parameter values are typically selected to have an adequate description of the available in vivo kinetic data (parameter fitting). Care must be taken when using these same available in vivo kinetic data for model evaluation, especially when they are limited, e.g. available only for one dose. Indeed, fitted parameter values may not adequately predict/describe the in vivo kinetics at other doses. The PPR Panel recommends to not derive PBK model parameter values solely based on in vivo kinetic data but to also use data from in vitro/in silico studies as starting point; the in vivo data can then be used for parameter values refinement or predictivity assessment of the model. In case several in vivo kinetic studies are available, it should be clearly indicated and justified

what data have been used for model development (parameterisation) and what data for model evaluation. Also, if certain studies are not used for PBK model parameterisation/evaluation, this should be duly substantiated as well.

The PPR Panel notes that evaluation of PBK model predictions against in vivo kinetic data is considered the standard for regulatory application (OECD, 2021; WHO, 2010). Such evaluations are typically performed using in vivo kinetic data on internal exposures to parent chemical (and to metabolites as well if they are described by the PBK model). It is acknowledged that if an in vivo ADME study is already available that does not contain information on internal exposure of parent chemical (and its metabolite(s)), performance of a full new ADME study according to OECD TG 417 (including quantification of parent chemical and metabolite(s) in blood, plasma, tissues) would require the use of a large number of animals (OECD, 2010). Rather, a targeted in vivo kinetic study may be performed with a limited number of animals, e.g. focussing on the time-dependent sampling of blood/plasma upon exposure to a relatively low and relatively high dose, as well as tissue sampling at the end of the study. Also, TK analysis can be included in regulatory toxicity studies (see Recommendations section). The PPR Panel underlines that, according to the latest OECD guidance on PBK modelling, other approaches such as application of read across using in vivo kinetic data of related substances can be used for PBK model evaluation (OECD, 2021). In case no in vivo kinetic data are available for PBK model evaluation, an assessment of the related uncertainties in the PBK model prediction is to be provided.

In case in vivo kinetic data are available from non-guideline studies, the PPR Panel considers essential a critical assessment of the quality of such studies. To obtain reliable kinetic data from these studies, adequate analytical assessment of the samples generated in the study is necessary (see Section 3). Other aspects to consider are the selection of the strain and age of the animals. In vitro kinetic studies in combination with some first predictive PBK modelling, may possibly provide relevant information for the design of the in vivo kinetic studies. For example, in vitro biotransformation studies with liver samples from young animals compared to adults (see, e.g. Kim et al., 2010; Liu et al., 2019) may indicate what life stage (young child or pregnant woman) is considered to provide the most conservative estimation of internal exposure and as such to be included in the PBK modelling.

5.7.3.2 | *General considerations on using in vivo kinetic data from humans*

Some kinetic studies with deliberate exposure in humans to pesticide active substances have been described in the scientific literature (e.g. Harada et al., 2016; Oerlemans et al., 2019; Ratelle et al., 2015). Although the use of in vivo kinetic data is generally recommended for PBK model evaluation (OECD, 2021; WHO, 2010), in line with the Regulation (EC) No 1107/2009, the PPR Panel does not recommend performing kinetic studies in humans for the parameterisation and evaluation of PBK models for pesticide active substances. However, in case such data have been already reported, these are to be considered.

Other human in vivo kinetic data to consider include human biomonitoring (HBM) data. Analytical results of pesticide residues and their metabolites in biological fluids from HBM studies can be of use for developing PBK models. HBM studies provide empirical data on the concentrations of pesticide residues and their metabolites in biological matrices such as blood, urine and tissues. Incorporating such data allows these models to better reflect human physiological conditions compared to models based solely on animal data or in vitro studies. However, unlike controlled in vivo kinetic studies, most HBM datasets lack information about external exposure, hampering the link between external and internal exposure. Nonetheless, some information may be used to derive PBK model parameter values and/or for model evaluation. For example, if information on paired concentrations in plasma and tissues is available (e.g. Laubscher et al., 2022), this information can be used to derive tissue:plasma partition coefficients if steady-state is reached. Also, regarding fetal exposure, paired maternal and cord blood samples from HBM studies may provide relevant information. The identification and quantification of pesticide metabolites in human samples can also help to understand metabolic pathways and the formation of potentially toxic metabolites. This information is essential for refining PBK models to include human-specific metabolic transformations as certain metabolites may be unique to humans or present in different proportions compared to other species (Pelkonen et al., 2023). Moreover, HBM data can reveal inter-individual variability in pesticide metabolism, influenced by factors such as age, sex, genetic polymorphisms and health status. If adequate information is available, PBK models can incorporate this variability to predict the range of possible human responses to pesticide exposure, enhancing their relevance for diverse populations.

5.7.3.3 | *General considerations on in vitro methods*

According to the ADME data requirements for the active substance laid down in Commission Regulation (EU) 283/2013, in vitro comparative studies should be performed that allow for the identification of species differences in metabolite formation. Currently, no test guideline is available related to the performance of such in vitro comparative metabolism studies. However, the PPR Panel published in 2021 a Scientific Opinion, describing a preferred approach on how to perform and report such studies for pesticide active substances and providing guidance on the interpretation of related data. Numerous other in vitro test systems have been described in the scientific literature that can be used to obtain insight into the toxicokinetics of chemicals and may support the parameterisation of PBK models. However, no harmonised test guidelines are currently available for these in vitro kinetic methods except for in vitro dermal absorption (OECD, 2004). Therefore, the PPR Panel considers it essential to have a critical assessment of the quality of the data from non-guideline in vitro studies for chemical-specific model parameterisation. Methods used should adhere to the OECD's GIVIMP (OECD, 2018). Specific considerations on critical aspects of the performance and assessment of in vitro kinetic studies are summarised below (for more information on possible methods, see Annex E).

Analytical aspects

Analytical methods used to determine the parent chemical and/or its metabolites formed during the test is an important component of the in vitro methods for identifying in vitro kinetic parameters (see Section 3). The choice of the method is always dependent on the chemical characteristics of the analyte (Tolonen & Pelkonen, 2015). The PPR Panel recommends specifying chemical purity for the test item and for any other reference chemicals, using as far as possible certified material with the highest available purity.

Parameters of the analytical method that should be always determined following established procedures as described by EC, EMA and FDA (EC, 2023; EMA, 2022; FDA, 2018) are the LOD and the LOQ. Effects of the matrix should be evaluated. The comparison between the LOQ and the nominal concentration at the start of the study helps in the study design to ensure the reliability of the obtained results. The PPR Panel recommends that the LOQ should be < 10% of the initial incubation concentration when clearance is measured by disappearance of the parent chemical.

Test system characterisation

Depending on the aim of the in vitro kinetic method (providing information on absorption, distribution, metabolism, excretion) different test systems can be used as described in the literature (Bessems et al., 2014; EFSA PPR Panel, 2021b; Gouliarmou et al., 2018; see also Annex E). Test system configuration (e.g. suspension, adherent monolayers, 3D) will dictate the length of time an in vitro system can maintain its physiological features, particularly its metabolic competence. Cells when used in a plated configuration can maintain their metabolic competence for longer duration and, therefore, they are the preferred choice when incubation of more than 4 h is necessary. It is recommended to perform some preliminary studies aimed to characterise the test system for transporter(s) presence and activity as well as metabolic competence, as described in the OECD GIVIMP document – Section 5 (OECD, 2018). In addition, the cell viability after isolation and/or thawing should be checked, which should meet pre-determined acceptability criteria and should preferably not be lower than 90%.

Reference chemicals/positive controls

Reference and control items, such as negative and positive controls, should be applied in general to verify the correct function of the in vitro test system. The purpose of the reference item(s) is to grade the response of the test system to the test item. Control items are used to control the proper performance of the test system. A vehicle (or solvent) control assures that a response does not originate from the applied solvent. Therefore, its concentration should be as low as possible, generally ranging between 0.1% and 1% (v/v), to avoid any artefactual effects. A negative control is an item for which the test system should not give a response, while a positive control is a chemical with a specific and well-known response (relevant to the aim of the test to be performed) and used to validate the performance of the test system and at the same time useful to grade for comparison the test item activity. The positive control may also be used as a reference item.

For a study that aims to provide kinetic data for PBK model parameterisation, a negative control may not be needed. However, it is essential to show that the vehicle used does not affect the kinetic parameters obtained.

Since the purpose of reference and control items is analogous, the definition of reference item may be regarded as covering the terms 'positive, negative and/or vehicle control items' (GIVIMP; OECD, 2018). Acceptance criteria based on historical data need to be developed and determined a priori.

Chemical-specific optimisation of experimental conditions

In order to design the in vitro kinetic study in a proper way, information on the physical–chemical properties and characterisation of the test item are required (GIVIMP; OECD, 2018): indeed they affect any non-enzymatic disappearance of the test item due to processes such as solubility, stability, volatility and non-specific binding, and consequently the actual concentration available for the cell uptake can be significantly lower than the 'nominal' added concentration (see Section 4 and Appendix B).

In line with OECD GIVIMP (OECD, 2018), the PPR Panel recommends determining solubility and volatility in the solvent used for the stock/working solutions and in the medium at the highest concentration and in the conditions of the test, as well as stability during storage and in the experimental conditions used.

The methods for checking stability during storage are described in OECD guidelines (OECD, 1981, 2007, 2016a); similar analytical methods can be used by measuring the test item pre- and post-incubation at 37°C in the cell medium.

Another process that can significantly reduce the freely available test item concentration in in vitro assays is the non-specific binding (EFSA PPR Panel, 2021b, Appendix B for details). The non-specific binding can be due to: (i) sorption to in vitro system components such as plastic labware and microtitre plates, particularly relevant for lipophilic test items (Kramer et al., 2015; Pomponio et al., 2015); (ii) binding to serum constituents in exposure medium, one of the most significant contributors to the reduction in the freely available concentration (Armitage et al., 2014; Kramer et al., 2015); (iii) adsorption to cell-attachment matrices (e.g. collagen or matrigel), when cells are seeded in sandwich configuration (Kramer et al., 2015). These aspects are to be considered when deriving kinetic parameter values from the studies, since if kinetic parameters are based on nominal concentrations applied, they may largely defer from the relevant parameters for PBK model parameterisation.

The concentrations that can be tested depend on the cytotoxicity: indeed in vitro TK testing should be carried out at non-cytotoxic concentrations (generally lower than 10% cell death) in order to have a fairly constant number of cells during testing. Indications on how to carry out the cytotoxicity test are given in the OECD GIVIMP document (OECD, 2018) and in the Scientific Opinion on comparative in vitro metabolism studies (EFSA PPR Panel, 2021a). At least three concentrations should be tested, but testing more concentrations is recommended. The total incubation time depends on the aim of the test, the biological test system used and its configuration, as described above. The timing of sampling is also another important chemical-specific aspect to consider, e.g. depending on the rate of metabolic conversion. The selection of the appropriate time points (at least three in addition to Time 0) usually needs to be optimised by performing some preliminary studies, on the basis of which the most adequate concentration of cells/enzymes can be selected (e.g. for intrinsic clearance (CLint) studies). In case the metabolism is very rapid, the quantity of enzyme can be reduced or vice-versa.

5.7.3.4 | *General considerations on in silico methods*

In silico methods provide important tools to support PBK model parameterisation for diverse ADME processes. Examples for the different ADME parameters are provided in Annex E. These overviews are not meant to be exhaustive, nor should the methods reported there be considered to always provide acceptable data. General aspects and considerations of applying in silico tools to derive data for PBK model parameterisation are provided in this sub-section. In silico models are generally subdivided into 3D/structure-based models and 2D/ligand-based models.

3D/structure-based models

These models allow studying the interactions between a chemical (ligand) and a protein and include molecular docking and molecular dynamic (MD) studies. Molecular docking applies scoring functions to estimate the delta $G_{binding}$, which can help determine whether a chemical is a substrate for a given biotransformation enzyme and/or transporter protein. To provide useful information, 'test items' need to be compared with 'reference items', i.e. chemicals that are known to be a substrate or a non-substrate. MD simulations provide insight into the kinetics of the chemical-target interaction and are computationally more intensive. Like docking, interpretation of the outcomes also relies on comparisons with 'reference items'. MD simulations have also been used to predict the impact of genetic polymorphisms and species differences in metabolism/transport for enzymes/transporters, as it considers the possible impact of the modified protein structure resulting from the genetic differences. While 3D/structure-based models are very informative from the mechanistic point of view, their capacity to make quantitative predictions is limited.

2D/ligand-based models

Well-known examples of 2D/ligand-based models are quantitative structure activity relationship (QSAR) models. 2D/ligand-based modelling is also typically applied for structural alert/expert rule and read-across assessments. A QSAR model is a mathematical model that relates quantitative measures of chemical structure to a physical property (also called a quantitative structure property relationship (QSPR)), a biological/toxicological effect or a kinetic process. For the toxicological hazard assessment of pesticide active substances, 2D/ligand-based models, including QSARs, are typically applied for the toxicological assessment of impurities and/or residue metabolites.

The PPR Panel considers that QSARs can be of use to support the derivation of parameter values for chemical-specific ADME processes, preferably in combination with information from in vitro and in vivo kinetic studies.

As with any QSAR model, QSARs for ADME processes should adhere to the OECD principles for QSAR validation (OECD, 2014), i.e. principles to improve transparency and acceptability, including: (1) a defined endpoint, (2) an unambiguous algorithm, (3) a defined domain of applicability, (4) appropriate measures of goodness-of-fit, robustness and predictivity and (5) a mechanistic interpretation (if possible).

According to the (Q)SAR assessment framework (QAF), model developers should provide a QSAR model reporting format (QMRF). However, the presence of a QMRF itself is not a sign of reliability and/or quality of the QSAR model. A QSAR user should provide a QPRF, to facilitate assessment of QSAR data by an evaluator. Elements covered in such a QPRF are (1) general information, (2) substance information, (3) information about the model and software used, (4) model predictions, (5) model input, (6) information about the applicability domain and limitations of the model, (7) a reliability assessment and (8) the purpose of the use. More information about the QAF, QMRF and QPRF can be found in OECD (2024) and Gissi et al. (2024).

5.8 | PBK model evaluation

The evaluation of a PBK model is a critical step to ensure its reliability and applicability for chemical risk assessment. As outlined in OECD Guidance No. 331 (OECD, 2021), model evaluation involves assessing the biological plausibility, structural correctness and predictive performance of the model. This includes verifying whether the model adequately represents key physiological and biochemical processes and whether its predictions align with observed in vivo data when available. The assessment should also consider model transparency and reproducibility, ensuring that assumptions, equations and input data sources are well-documented. OECD Guidance No. 331 encompass an 'evaluation checklist' including specific

questions for the model's implementation and the assessment of its validity (OECD, 2021). The PPR Panel recommends using this evaluation checklist for the PBK modelling approach used in QIVIVE cases.

5.8.1 | Evaluation of model predictivity

PBK model predictivity assessment refers to the evaluation of a model's ability to accurately reproduce empirical in vivo kinetic data. It is a key aspect of model validation, ensuring that the model's predictions align with observed biological data under relevant exposure conditions. This assessment typically involves goodness-of-fit analysis, where predicted concentration-time profiles of chemicals in different tissues or plasma are compared against measured experimental data. Common predictivity metrics include statistical goodness-of-fit measures (e.g. R^2, mean squared error, Akaike information criterion), fold error analysis (assessing the deviation between predicted and observed values) or visual predictive checks (where simulated data distributions are overlaid with experimental observations).

Unlike other aspects of model evaluation (e.g. biological plausibility, theoretical consistency, uncertainty analysis and sensitivity assessment), predictivity assessment is uniquely dependent on the availability of empirical in vivo kinetic data for direct comparison. This step strengthens confidence in the PBK model's applicability for risk assessment and regulatory decision-making.

It is important to note that predictivity of a given model output does not allow to presume predictivity for other model outputs.

5.8.2 | Uncertainty and sensitivity analysis

Uncertainty and sensitivity analyses are critical components of PBK model evaluation in order to understand the reliability and robustness of model predictions. Uncertainty analysis quantifies the overall uncertainty in model outputs arising from uncertainties in input parameters, whereas sensitivity analysis identifies how much each input parameter contributes to this uncertainty. In this context, variability represents an irreducible source of uncertainty that must be accounted for in model interpretation. The overall uncertainty of the model outcome (quantitative or qualitative) can be incorporated into the weight of evidence analysis (see Section 7).

Sensitivity analysis relies on screening methods, such as the Morris method. They are used to rank model parameters based on their relative importance while requiring relatively few model runs. These methods provide qualitative information, but exact contributions to output variability are not quantified. Screening is particularly valuable in the early stages of sensitivity analysis, helping to identify non-influential parameters that can be excluded from more computationally intensive analyses (Morris, 1991; Saltelli et al., 2004).

When parameter interactions are expected, global sensitivity analysis is preferred, as it evaluates all model parameters simultaneously (Hsieh et al., 2018). Global sensitivity analysis methods, such as Sobol's variance-based decomposition, estimate three key sensitivity indices:

- Main effect (First-order index) – Measures the proportion of output variance attributed to a single parameter, indicating the potential reduction in variance if the parameters were known precisely.
- Interaction effect – Captures how much of the output variation arises from interactions between multiple parameters.
- Total effect (Total-order index) – Represents the combined influence of an individual parameter and its interactions with all other parameters, quantifying the residual variance that would remain if only that parameter was allowed to vary.

A structured workflow for uncertainty and sensitivity analysis, as recommended by OECD Guidance 331 (OECD, 2021) and implemented in TKPlate (Bossier et al., 2023), involves:

1. Defining parameter variability – All parameters are treated as variables. Parameter ranges may be set using the 5th and 95th percentiles of their distributions, or through uniform ranges based on reasonable assumptions (e.g. lower bound = mean/2, upper bound = mean × 2).
2. Performing Morris screening – To identify the most influential parameters, allowing exclusion of non-influential ones from further detailed analysis.
3. Conducting global sensitivity analysis on selected parameters – Presenting first-order, total-order and interaction effects using Lowry plots for clear visualisation of parameter importance and interactions.

By systematically applying uncertainty and sensitivity analysis, PBK models can be refined for increased reliability, reduced computational complexity and improved regulatory acceptance.

6 | CONSIDERATIONS OF ANIMAL/HUMAN METABOLITES

The OECD DNT IVB document (OECD, 2023a) indicates that '*Many in vitro test systems, including the DNT IVB, have minimal or unknown metabolic capacities compared to liver.*' Appendix B of that document provides further details on DNT IVB

test systems, indicating that their biotransformation capacity has either not been characterised or is limited to mRNA expression of selected biotransformation enzymes. The DNT IVB test systems are expected to have low metabolic capacity, and additional quantitative insight into their biotransformation potential and comparison with the in vivo conditions would be useful. The PPR Panel recommends extending the characterisation of the biotransformation capacities of the DNT IVB test systems to determine whether test items can be metabolised in these systems (see more considerations in Section 10).

Assuming negligible biotransformation by the DNT IVB test systems, one can ascribe the observed effects (or no effects) to the parent chemical added to the system providing that preliminary testing demonstrates that the parent chemical is stable and not subject to abiotic degradation in the experimental conditions tested (see Section 4). For QIVIVE, this may be the simplest approach as one would not need to distinguish the contribution of parent chemical versus metabolite(s) in the test system to the effect observed. When testing the parent chemical only, an obvious drawback of lack of biotransformation capacity of the test systems is that possible toxicity of biotransformation products remains undetected. Even if the test systems would mimic tissues with a low biotransformation competence in vivo, stable metabolites formed in the maternal and/or fetal liver or in other tissues may reach the developing brain, and no insight into their possible toxicity is obtained when only testing the parent chemical in vitro. This is a well-known limitation of test systems lacking biotransformation capacity. Currently, insight into the role of metabolites in the in vivo DNT of chemicals is limited (see Section 10.1).

Inclusion of metabolic activation systems (e.g. liver S9 fraction with appropriate co-factors) in testing, as described in various regulatory in vitro toxicity tests (e.g. the bacterial reverse mutation test – OECD, 2010, the in vitro mammalian cell gene mutation test – OECD, 2016b and the in vitro micronucleus test – OECD, 2023b) can help identify toxic metabolites. However, considerations on the addition of metabolic activation systems to the DNT IVB are not described in the OECD document (OECD, 2023a) and it is not known whether these test systems are compatible for co-incubation with metabolic systems. Furthermore, conducting QIVIVE using data from systems with added metabolic components is not straightforward as time-dependent changes in parent chemical and metabolite concentrations complicate identification of the causative agent(s). In order words, selection of a relevant in vitro exposure metric would be very difficult, if possible at all.

In theory, toxicity may also be caused by a local formation of a metabolite, which may especially be of relevance in case this is a reactive metabolite, being short-lived, difficult to be analytically detected and acting at the site of formation. Although such examples of DNT chemicals demonstrating this may not be available, uncertainty of the QIVIVE of DNT IVB data may be reduced by increasing knowledge on the capacity of the metabolic capacity of DNT IVB test systems as considered relevant for the (local) in vivo situation (see for related recommendations Section 10).

To assess the bioactivity of a metabolite (e.g. those formed by the maternal or fetal liver and able to reach the developing brain) and their possible contribution to DNT, the most straightforward approach would be to test them directly in the DNT IVB assays, if available. Also, read-across approaches can be considered. Although it is outside the scope of this Scientific Opinion, the PPR Panel recommends developing a strategy on metabolites testing within the DNT IVB (see for more considerations Section 10.2). So far, few data have been published on the testing of metabolites in this context (e.g. chlorpyrifos oxon, a metabolite of chlorpyrifos – Blum et al., 2023).

Although there is limited information on the role of metabolites in chemical-induced DNT, various examples have been reported in which toxicity is caused by a metabolite and not by the parent chemicals (e.g. various glycol ethers and organophosphates). If, in such cases, only the parent chemical would have been tested in in vitro assays without biotransformation capacity, and QIVIVE would be based on related toxicity data of the parent chemical only, the resulting OED may not be informative or sufficiently conservative. Also, if both parent and (major) metabolite(s) are active, QIVIVE only based on the parent may not provide a sufficiently conservative OED. Therefore, in the absence of toxicity data on metabolites in the DNT IVB, the related uncertainty is to be addressed in the uncertainty analysis and considered in the overall weight of evidence analysis.

7 | IDENTIFICATION OF QIVIVE-RELATED UNCERTAINTIES AND APPLICATION OF QIVIVE OUTCOME INTO WEIGHT OF EVIDENCE ANALYSIS

7.1 | Identification of QIVIVE-related uncertainties

EFSA published a guidance document on uncertainty analysis in scientific assessments (EFSA Scientific Committee, 2018a). Sources of uncertainty pertain to all input variables related to the toxicokinetic and toxicodynamic processes involved in an assessment. The present Scientific Opinion focuses on the uncertainties related to the QIVIVE and does not provide a full uncertainty analysis of the assessment of DNT hazards and related risks for the pesticide active substance, which is out of its scope. Similarly, the current document focuses on kinetic aspects of QIVIVE and not on in vitro toxicodynamic considerations.

The earlier sections described extensively the critical steps of QIVIVE and associated uncertainties, and this section summarises the most critical QIVIVE-related uncertainties. For related background information, readers are referred to the relevant sections. The PPR Panel recommends organising the information on the uncertainties of the QIVIVE related to main building blocks of the QIVIVE, i.e. (1) the in vitro-based exposure metric, (2) the PBK modelling-predicted internal exposure metric and (3) linking of the PBK modelling-based internal exposure metric to the in vitro exposure metric.

7.1.1 | In vitro exposure metric

Uncertainties related to the in vitro exposure metric used for the QIVIVE relate to possible lack of, or limited information on, the in vitro distribution kinetics and the related unbound in vitro concentration and/or cell-associated concentration, i.e. the recommended in vitro exposure metrics for the QIVIVE (see Section 4). Uncertainties in the estimation of the free and/or cell-associated concentration depend on several aspects that must be considered. These include the availability of experimental data from an in vitro distribution kinetic study. If available, the quality of the study is to be considered for the uncertainty analysis. In Section 4.2 it is indicated that if no experimental in vitro distribution kinetic study is available, in silico mass balance models may be used to estimate unbound medium and cell-associated concentrations at steady-state. Currently, these models have not been applied to and evaluated for their prediction of in vitro distribution kinetics in the in vitro assays of the DNT IVB. It remains to be elucidated which model for what chemical and what in vitro assay in the DNT IVB provides accurate predictions. Application of different in silico distribution models may provide relevant information for the uncertainty analysis, e.g. when QIVIVE is based on the nominal concentration.

Another important source of uncertainty for the determination of the in vitro exposure metric is whether an effect in the in vitro test system is caused by the parent chemical itself that is added to the test system, a breakdown product and/or a metabolite. In case an experimental in vitro distribution kinetic study is performed, and the parent is mainly identified (with an adequate recovery), the related uncertainty is considered low and the effect can be attributed to the parent chemical. In the absence of an in vitro distribution kinetic study, and the parent chemical breaks down to other molecules, which can be due to abiotic factors and/or by enzymatic biotransformation, there is uncertainty regarding the causative agent of the observed effect and, consequently, the selection of the relevant in vitro exposure metric. This uncertainty can be reduced based on information on the stability of the test item in the test system (as measured in an in vitro distribution kinetic study), as well as by knowledge on the test systems' capacity for biotransformation. As indicated in Section 6, information is limited on the biotransformation capacity of the test systems in the DNT IVB.

Other uncertainties relevant for any QIVIVE of DNT IVB data relate to possible differences in metabolic competence between the in vitro system and the in vivo developing brain. If such differences exist, they introduce uncertainties concerning the local formation of (reactive) metabolites in vivo that may be responsible for/play a role in the toxicity. To reduce related uncertainties, the PPR Panel recommends characterising the biotransformation capacity of the in vitro test systems and benchmarking them against the in vivo situation (see Section 10.2).

Another source of uncertainty in the interpretation of QIVIVE results of DNT IVB data relates to possible in vivo exposure of the developing brain to (stable) metabolites formed in other tissues (e.g. in liver), which may not be formed (or only to a limited extent) by the cells used in the DNT IVB test systems. These metabolites may reach the developing brain and contribute to DNT. If such metabolites are not tested in the DNT IVB test systems, the QIVIVE outcome based on only parent may not represent potential DNT activity of the parent and its biotransformation metabolites that are formed in test species and, more relevant, in humans.

Besides the uncertainty of the in vitro exposure metric, the quality of the DNT IVB studies should be considered in the QIVIVE assessment. Various tools are available to assess the reliability of such toxicity studies (e.g. OHAT NTP, SciRap, ToxRTool). Although in vitro exposure considerations are extensively discussed in this Scientific Opinion (Section 4), considerations for a full reliability assessment are outside the remit of this work.

7.1.2 | Predictions of internal exposure metric

Uncertainties related to linking an external exposure metric to an internal exposure metric pertain to the PBK modelling process. As indicated in Section 5.8.2, sensitivity and uncertainty analyses are critical aspects in both the development and application of PBK models. Important aspects of such assessments are described in that section and more details can be found in the OECD guidance document (OECD, 2021).

For the QIVIVE of DNT IVB data, specific attention should be given to whether adequate in vivo kinetic data were available for PBK model evaluation. The presence or absence of such data significantly affects the level of uncertainty for the estimation of the internal exposure metric. In the absence of adequate in vivo kinetic data for PBK model evaluation, the uncertainty associated with PBK model predictions is considered relatively high. It is of interest to note that a few studies have evaluated the predictive capacity of PBK models based on solely in vitro and in silico input data for estimating internal plasma concentration (C_{max}) (Punt, Louisse, Beekmann, et al., 2022; Punt, Louisse, Pinckaers, et al., 2022; Wambaugh et al., 2015). Insights from these studies may help in the quantitative assessment of the uncertainties associated with such PBK models.

The uncertainty analysis should not only focus on the sensitive parameters in the PBK model, since in absence of an evaluation against in vivo data, critical kinetic process may not be identified if they are not included in the PBK model description.

7.1.3 | Linking of the PBK modelling-based internal exposure metric to the in vitro exposure metric

As indicated in Section 4, the PPR Panel recommends focusing the QIVIVE based on linking in vitro concentration to either the C_{max} or C_{ss} in blood or plasma (scenario A), or to the corresponding concentration in target tissues (scenario B). However,

it also recommends performing QIVIVE-based on other exposure metrics (e.g. AUC) to explore how the related QIVIVE outcome would differ when different metrics are applied. There is uncertainty how well in vitro data (especially when applying acute/short-term exposure) capture effects induced in vivo following (sub)chronic/continuous exposure. This can be considered an inherent uncertainty in QIVIVE but may be partly addressed considering QIVIVE based on AUC as exposure metric.

Differences in QIVIVE outcomes when based on C_{max} or C_{ss} versus AUC should be explicitly described and used for the uncertainty analysis of the QIVIVE. These comparisons help inform the reliability and the interpretation of the QIVIVE results.

7.1.4 | Reporting of QIVIVE-related uncertainties

A structured reporting table can be used to summarise the results of the uncertainty analysis and the identified data gaps. Each identified uncertainty should be reported, described and quantified, when possible.

For each aspect, an evaluation should be conducted for each chemical to determine whether the uncertainty is considered low, medium or high. The expected impact of each uncertainty on the QIVIVE outcome should be considered. Some recommendations for the QIVIVE on reporting are provided in Section 8. In addition to chemical-specific uncertainties, there are also inherent uncertainties associated with the QIVIVE approach itself. These include uncertainties related to the biological relevance of effects observed in DNT IVB assay(s), specifically whether such effects are predictive of potential adverse human effects in vivo. Additional uncertainties relate to differences in exposure duration in vitro in the DNT IVB versus real-life during pregnancy and early postnatal period in vivo. Such uncertainties should be considered in the weight of evidence analysis of the full DNT assessment of the chemical of interest (see Section 7.2).

Ideally the main sources of uncertainty in the QIVIVE should be quantitatively transparent, i.e. to which extent is the overall uncertainty dominated by uncertainty in the in vitro exposure metric, uncertainty in the PBK model predictions, including the (measured) PBK model parameter uncertainty and uncertainty about human variability. Such stratified uncertainty information may help to decide on acceptable uncertainty in the overall output. The lower the uncertainty in the in vitro exposure metric and PBK model output, relative to the uncertainty in human variability, the more acceptable the model output may be.

7.2 | Application into weight of evidence analysis

The QIVIVE outcome contributes to the overall body of evidence considered in the hazard and risk assessment of the pesticide active substance. Information on the principles of weight of evidence (WoE) assessments, including how to incorporate QIVIVE outcomes is provided here.

WoE approaches are critical to the risk assessment process, particularly in chemical risk assessment, with regards to hazard identification and characterisation. According to EFSA's guidance, WoE is defined as a structured process in which evidence is integrated to determine the relative support for possible answers to a question, most often defined in the problem formulation. This process takes into account the biological relevance of the observed effects and associated uncertainties (EFSA Scientific Committee, 2017a, 2017b, 2018a, 2018b). The WoE framework is flexible, allowing assessors to use the most appropriate approach depending on the context and purpose of the assessment. It may include simple description (narrative or tabular summaries), semi-quantitative methods (categorising confidence as low, moderate, high) or quantitative methods (probabilistic scale) and tiered approaches that are fit for the purpose of any individual assessment.

The WoE approach requires three basic steps:

1. assembling the available evidence into lines of evidence,
2. weighing the evidence,
3. integrating the evidence.

Weighing evidence requires three basic considerations, i.e. (a) reliability as the extent to which the information comprising a piece or line of evidence (LoE) is correct, (b) relevance as the contribution of a piece or LoE to answer a specified question, if the information comprising the LoE is fully reliable and (c) consistency as the extent to which the contributions of different pieces or LoE to answering the specified question are compatible. EFSA guidance also includes case studies illustrating the WoE approach, including in silico tools (EFSA Scientific Committee, 2018a).

For chemicals, the WoE approach for assessing hazard information requires systematic data collection and organisation into LoEs (e.g. mode of action, adverse outcome, critical effect, target organs) at different biological levels (i.e. molecular, cellular, organ, whole organism). These LoE are then weighed and integrated to support hazard conclusions.

The WoE approach is particularly relevant for the integration of NAMs, such as results of in vitro assays, into chemical hazard and risk assessment. This has been addressed in EFSA's guidance document on risk assessment of combined exposure to multiple chemicals and the guidance on scientific criteria to group chemicals into assessment groups using hazard-driven criteria for both toxicokinetic and toxicodynamic information (EFSA Scientific Committee, 2021).

8 | PERFORMING AND REPORTING THE QIVIVE APPROACH

In the preceding sections of this Scientific Opinion, different features of the QIVIVE general approach have been discussed together with recommendations and considerations provided. Based on these discussion points, the current section presents a framework for performing and reporting QIVIVE of DNT IVB data while reminding readers to consult the OECD relevant guidances for specific details regarding the DNT IVB assay characteristics and PBK modelling.

The 4 building blocks of the framework focus on (1) the in vitro exposure metric selection, (2) PBK modelling (with related internal exposure prediction), (3) the QIVIVE itself (linking in vitro exposure metric(s) to external equivalent exposure(s)) and (4) uncertainty analysis for the QIVIVE (Figure 8). For each of these steps, detailed information is to be provided.

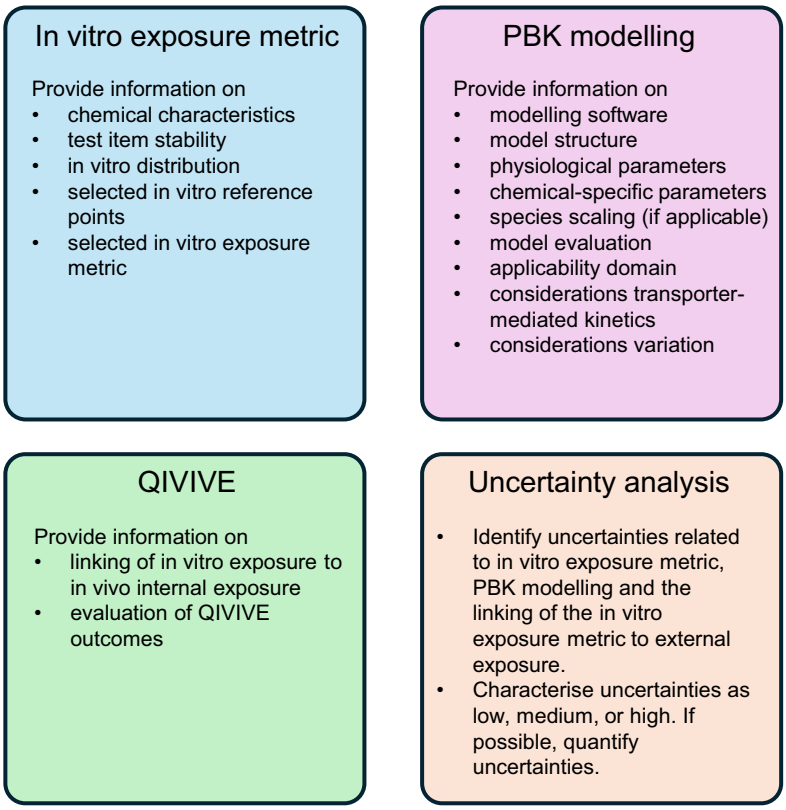

FIGURE 8 Schematic overview of building blocks of the proposed QIVIVE framework and information to be provided on the different aspects of the QIVIVE.

Related to the in vitro exposure metric selection, information on the physicochemical characteristics of the test item should be provided as well as the stability of the test item, and the in vitro distribution kinetics of the test item in the test systems. Substantiation should be provided on the in vitro reference points selected for the QIVIVE and the selected in vitro exposure metric(s) (unbound concentration medium, nominal concentration medium and/or cell-associated concentration). See Section 4 of this document for considerations for in vitro exposure metric selection.

For the PBK modelling, information should be provided on the modelling software/tool used, the PBK model structure selected and the physiological and chemical-specific parameters applied in the PBK model. If species scaling is applied, a clear substantiation and insight into related uncertainties should be provided. A thorough model evaluation should be performed, including evaluation of the model predictions and a related uncertainty and sensitivity analysis. Substantiation as to whether the applied PBK model is adequate for the specific QIVIVE case should also be provided, i.e. species, life stages, exposure routes, dose ranges, reliable predictions from the PBK model for the intended use. Furthermore, considerations are to be provided for possible transporter-mediated kinetics and variation in kinetic parameters and their impact on the PBK model predictions. The PPR Panel recommends using the reporting templates of the OECD guidance (2021). See Section 5 of this document for considerations for PBK modelling.

For the actual QIVIVE, a clear description and substantiation should be provided for the selected in vitro exposure metric and how it is linked to the internal exposure metric in the PBK model. The reader should refer to Section 4 of this document for considerations to substantiate the link between the in vitro exposure metric and the internal exposure metric. Furthermore, an evaluation of the QIVIVE outcomes should be provided for the reverse dosimetry-based and/or forward dosimetry-based approaches that are applied. For more related information, please refer to Section 5.5.

For the different aspects of the QIVIVE, a table with a description of the uncertainties should be provided for the specific QIVIVE case, including each uncertainty identified, whether it is considered to be low, medium or high, and substantiation on the expected global impact of these uncertainties on the outcome of the QIVIVE. While such ordinal scales can serve as a pragmatic starting point, their qualitative nature may introduce subjectivity. Therefore, where feasible, they should be complemented with broader methods (e.g. quantitative sensitivity analyses, probabilistic approaches, scenario analysis) to improve transparency, reduce ambiguity and strengthen the interpretation of uncertainties. The uncertainty table should focus on the features summarised in Section 7.1, as well as any other specific aspect that is deemed relevant on a case-by-case basis.

In Annex F, a related reporting template is provided, and it is recommended that this template is used for reporting the QIVIVE case and included in the corresponding study report.

9 | CONCLUSIONS

This section summarises the main conclusions in relation to the ToRs of this Scientific Opinion and refers to the relevant sections where more details can be found.

ToR 1: Minimal data requirements for chemical-specific input parameter values for a fit for purpose PBK model applied for the QIVIVE and quality criteria for kinetic data.

It is concluded that a minimal set of chemical-specific parameters is required for the application of PBK models in QIVIVE. These include fraction absorbed, intestinal uptake rate, fraction unbound in plasma, tissue:plasma partition coefficients and hepatic biotransformation rates (intrinsic clearance or K_m and V_{max}). For higher tier models, additional parameters such as transfer across the blood:brain barrier, placenta and/or into milk may be necessary. Regarding quality criteria for in vitro kinetic studies, key aspects include analytical methods (including appropriate sample preparation (extraction) and assessment of matrix effects, and application of validated analytical methods), test system characterisation, inclusion of reference/control chemicals and chemical-specific optimisation of experimental conditions. Adherence to OECD GIVIMP is critical. For in silico approaches, adherence to OECD QSAR principles and robust reporting frameworks is essential. Relevant recommendations and detailed criteria are described in Sections 3 and 5.7.

ToR2: The needed in vitro biokinetic studies to determine the relevant in vitro exposure parameters for the QIVIVE of the assays included in the DNT IVB.

Relevant in vitro exposure metrics for QIVIVE include the unbound medium concentration and the cell-associated concentration. For situations under which the unbound medium concentration is not available, the nominal concentration may be used (depending on chemical characteristics).

For reliable quantification of the chemical in the test system, critical analytical aspects described in Section 3.3 should be adhered to, including appropriate sample preparation (extraction) and assessment of matrix effects and application of validated analytical methods.

In silico approaches and/or analytical measurements to assess the distribution of the test item within in vitro test systems (biokinetic studies) are detailed in Section 4 and Appendix B.

ToR3: A preferred approach to perform and report the QIVIVE

A framework is proposed in Section 8 to perform and report QIVIVE, following the main concepts discussed in this document. These include selection of the in vitro exposure metric (Section 4), PBK modelling aspects (including PBK model selection, parameterisation and evaluation), evaluation of QIVIVE outcomes (Section 5) and performance of an uncertainty analysis (Section 7). The related reporting template is available in Annex F.

ToR4: Main potential sources of uncertainties of the proposed QIVIVE for pesticide human health risk assessment.

The main potential sources of uncertainties in QIVIVE, summarised in Section 7, include (1) the in vitro exposure metric selection, (2) PBK modelling (with the related prediction of internal exposure metric) and (3) linking the in vitro exposure metric to the internal exposure metric. Uncertainties may differ not only between chemicals but also due to factors such as the type and quality of the in vitro assay, the specific QIVIVE objective (e.g. point of departure derivation vs. potency ranking), the exposure scenario considered (e.g. acute vs. chronic, dietary vs. occupational), the PBK model structure and parameterisation and the reliability of input data. These factors can influence both the magnitude and the nature of uncertainty and should therefore be evaluated on a case-by-case basis. Inherent uncertainties of the QIVIVE relate to the biological relevance of effects observed in DNT IVB assay(s), specifically whether such effects are predictive of potential adverse human effects in vivo. Considerations to the possible role of metabolites in DNT are provided in Section 6, and specific recommendations for addressing related knowledge gaps and reducing uncertainty in Section 10.

Altogether, this Scientific Opinion outlines the minimum data requirements, considerations for analytical methodologies and in vitro distribution kinetics, as well as a harmonised reporting framework necessary to support PBK modelling-based QIVIVE of DNT IVB data. It further identifies key sources of uncertainty and offers recommendations for their evaluation, thereby promoting a more reliable and transparent application of DNT IVB assays in pesticide human health hazard and risk assessment.

10 | RECOMMENDATIONS

This section provides some key recommendations to improve QIVIVE of DNT IVB data with the purpose of reducing associated uncertainties.

10.1 | Insight into the role of metabolites in DNT

As indicated in Sections 6 and 7, uncertainties need to be addressed related to the possible contribution of metabolites to the DNT associated with chemical exposure. A systematic analysis about the available in vivo and in vitro evidence for pesticide active substances and any other chemicals causing DNT due to biotransformation is recommended to understand the dimension of the uncertainty.

10.2 | Assessment of biotransformation capacity of DNT IVB assays

The various tests included in the DNT IVB employ different cell types of both human and rodent origin. However, for many of these test systems, the biotransformation capacity, as well as the expression and activities of transporters regulating the influx/efflux of both the parent chemical and its potential metabolites, is mostly unknown.

The PPR Panel recommends gathering existing knowledge from the open literature on the properties of the test systems, as well as the in vivo characteristics of the corresponding cell types and developmental stage the test system corresponds to. Lack of metabolic capacity is not necessarily a limitation, particularly if the equivalent in vivo cells at that relevant developmental stage also lack (or have very limited) metabolic activity. In such cases, the toxicologically relevant molecule is the parent chemical, since no metabolite would be formed in situ. However, stable metabolites formed in the maternal or fetal liver may reach the developing brain and exert toxic effects in vivo.

The PPR Panel also recommends compiling existing knowledge from open literature (retrieved by means of literature searches, according to EFSA, 2010) and/or from databases (e.g. ADME data in US-EPA dashboard,[23] ECVAM databases[24] or OpenFoodTox[25]) on the test item itself (when it is an existing one subject to renewal or a molecule already authorised for other purposes or outside the EU market) or on structurally similar test items (applying the principle of read across) before planning any testing. This information will allow to understand whether the parent and/or metabolite is estimated to be the toxicologically relevant molecule.

The PPR Panel further recommends characterising the metabolic capacity of the test systems, when not yet done, by exposing them (or their homogenates) to probe substrates of key enzyme families (e.g. CYP isoforms, uridine 5'-diphosph o-glucuronosyltransferases (UGTs), sulphotransferases (SULTs), glutathione-S-transferases (GSTs), carboxylesterases (CEs)). Particular attention should be given where these enzymes are known or suspected to metabolise the test substance. While gene or protein expression data can be informative, enzyme activity data are considered most relevant for functional characterisation. The same applies to transporter proteins whose presence and activity (e.g. by incubating the test system with known substrates), can inform on the role of active transport in determining cellular exposure to the parent and/or metabolites across the cell membrane.

10.3 | Strategy for testing metabolites in the DNT IVB and related performance of QIVIVE

The PPR Panel recommends developing a strategy for assessing the activity of relevant metabolites in the DNT IVB. This includes both metabolites formed locally in vivo (but not in vitro) and stable metabolites formed elsewhere in the body (e.g. maternal or fetal liver) and transported in vivo (but absent in vitro). In such a strategy, information on the metabolites formed in the regulatory in vivo ADME studies and data from in vitro comparative metabolism studies should be used to identify candidate metabolites for testing in the DNT IVB. Also, possible insight into structure activity relationships may be used to support selection of metabolites for testing.

Metabolites should be selected for QIVIVE inclusion based on their relative abundance and toxicological relevance. Where appropriate, a combined QIVIVE approach may be applied, accounting for both parent chemical and its metabolites.

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

[24] https://data.jrc.ec.europa.eu/dataset/8dfd6941-c011-4fbc-a247-aebaa3eb6b09.

[25] https://www.efsa.europa.eu/en/discover/infographics/openfoodtox-chemical-hazards-database.

In such cases, the PBK model should provide an adequate description of the toxicokinetics of the parent chemical and the metabolite(s) of interest. Zhao et al. (2021) used such an approach determining in vitro toxic equivalency factors (TEFs) for diazinon and its metabolite diazoxon for acetylcholinesterase inhibition and applied these as internal TEFs for the QIVIVE.

10.4 | Assessment of uncertainty of DNT IVB data due to interspecies differences

The PPR panel recommends considering uncertainties for interspecies differences in ADME when including NAM-based approaches in DNT assessment (and toxicity in general), as important information to decide on the acceptability of uncertainty from QIVIVE-based approaches. Data from in vitro comparative metabolism studies can support qualification and quantification of interspecies differences in hepatic metabolism between test species and humans. However, the overall uncertainty in species differences in ADME is not only limited to this process. Indeed, besides the metabolic capacity of the test systems (see above), other factors to be considered include the different timing of maturation during pregnancy, the placenta structure and BBB maturation, depending on the exposure window. There is limited information about species differences in fetal exposure related to these aspects, and therefore the PPR panel recommends collecting data on this topic.

10.5 | Update of OECD test guideline 417 to provide adequate data for PBK model parameterisation and evaluation

In line with previous recommendations (EFSA, 2024), the PPR Panel recommends updating the OECD TG 417 for in vivo toxicokinetic studies, by including the measurement of internal concentrations (at least in blood and plasma, and preferably also in tissues) of the parent chemical and the major metabolites over time. These measurements should supplement total radioactivity data. Such information would allow a better parameterisation and evaluation of PBK models, providing greater insight into the kinetic behaviour of chemicals without the need for additional animal studies.

10.6 | Inclusion of kinetic analyses in general toxicity studies

The PPR Panel recommends including kinetic analyses of parent and main metabolites in general toxicity studies, such as 90-day repeat-dose study, developmental toxicity study, chronic toxicity study. Such information would allow a better parameterisation and evaluation of PBK models, providing greater insight into the kinetic behaviour of chemicals without the need for additional animal studies.

10.7 | Performance of case studies

The PPR Panel recommends performing DNT IVB QIVIVE case studies for pesticide active substances, following the approach described in this Scientific Opinion.

10.8 | Inclusion of in vitro kinetic studies in data requirements

The PPR Panel recommends including in vitro kinetic studies (such as hepatic CL_{int}, fraction unbound plasma) using test systems for humans and relevant test species in the data requirements for pesticide active substances.

10.9 | Development of test guidelines for in vitro kinetic methods

The PPR Panel recommends development of test guidelines for in vitro kinetic methods, including for in vitro comparative metabolism studies (being already a data requirement), hepatic CLint and fraction unbound plasma.

10.10 | Development of guidance for scaling of in vitro kinetic data to in vivo PBK model parameters

The PPR Panel recommends developing guidance for the scaling of in vitro kinetic data to in vivo PBK model parameters. In vitro distribution kinetics for in vitro ADME test systems should also be considered.

10.11 | Performance of studies to support development and evaluation of in silico models predicting test item distribution in in vitro test systems of the DNT IVB

The PPR Panel recommends characterising test systems from the DNT IVB with regards to parameters required for the parameterisation of in silico models predicting test item distribution in the in vitro test systems. Also, studies should be performed to evaluate these in silico models for pesticide active substance distribution in the DNT IVB test systems.

ABBREVIATIONS

AAOEL	acute acceptable operator exposure level
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, excretion
AOEL	acceptable operator exposure level
AQC	analytical quality control
ARfD	acute reference dose
AUC	area under the curve
BBB	blood:brain barrier
BCRP	breast cancer resistance protein
BMC	benchmark concentration
BMD	benchmark dose
BMDL	benchmark dose lower confidence limit
BMR	benchmark response
CE	carboxylesterase
DNT	developmental neurotoxicity
EC50	half maximal effective concentration
EMA	European Medicine Agency
EU MACP	EU-coordinated multiannual control programme
FDA	US Food and Drug Administration
GC	gas chromatography
GIVIMP	good in vitro method practices
GST	glutathione-S-transferase
HBGV	health-based guidance value
HBM	human biomonitoring
IATA	integrated approaches to testing and assessment
IS	internal standard
IVB	in vitro battery
LC	liquid chromatography
LLE	liquid–liquid extraction
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LoE	line of evidence
LOQ	limit of quantification
MANCP	multiannual national control programmes
MD	molecular dynamic
MIE	molecular initiating event
MOA	mode of action
MS	mass spectrometry
NOAEL	no observed adverse effect level
NOEC	no effect concentration
OED	oral equivalent dose
PBBK	physiologically based biokinetic
PBK	physiologically based kinetic
PBPK	physiologically based pharmacokinetic
PBTK	physiologically based toxicokinetic
PgP	P-glycoprotein
POD	point of departure
PPR	EFSA Panel on Plant Protection Products and their Residues
QC	quality control
QIVIVE	quantitative in vitro to in vivo extrapolation
QMRF	QSAR model reporting format
QSAR	quantitative structure activity relationship
QSPR	quantitative structure property relationship
QuEChERS	quick, easy, cheap, effective, rugged, and safe

RP	reference point
SPE	solid-phase extraction
SULT	sulphotransferase
TEF	toxic equivalency factor
ToR	Terms of Reference
TRV	toxicological reference value
TTR	total radioactive residue
UGT	uridine 5'-diphospho-glucuronosyltransferase
WG	working group
WoE	weight of evidence
WPHA	OECD's Working Party on Hazard Assessment

GLOSSARY

Biologically effective dose (concentration) The biologically effective dose or concentration refers to the concentration of a substance that is responsible for producing a (toxicological) effect to cells or tissues, i.e. the dose/concentration that is available to interact with the cells/cellular targets. Also known as the dose (concentration) at the target site (e.g. DNA, cytoplasm or membrane receptors) in cells or tissues that causes a (toxicological) effect (e.g. µmol/µmol receptor; Groothuis et al., 2015).

Cell-associated concentration The cell-associated or cellular concentration is the concentration of a test chemical in the cells (unbound and bound) of an in vitro test system. It can be estimated using in silico mass balance tools or by analytical measurements in in vitro test systems.

External exposure metric External (oral) exposure, e.g. a toxicological reference value or a no observed adverse effect level, typically expressed in mg/kg bodyweight (per day).

Forward Dosimetry Forward dosimetry refers to the process of predicting or estimating the internal concentration of a substance, e.g. in blood/plasma or target tissues or organs, based on given external exposure information (e.g. oral, inhalation or dermal exposure) applying PBK models.

Fraction absorbed Fraction of the added dose that is systemically available upon exposure. In EU pesticide active substance assessment, the fraction absorbed upon oral exposure is typically obtained, referred to as the oral absorption value.

Internal exposure metric Internal in vivo exposure, such as a concentration in plasma or in an organ or tissue.

In vitro exposure metric Exposure metric in in vitro test systems (of the DNT IVB), such as an unbound concentration in the medium or a cellular (cell-associated) concentration.

In vitro reference point In the context of this document, the in vitro reference point (also called in vitro point of departure) refers to the concentration or dose of a substance obtained from an in vitro study used for the QIVIVE.

Nominal concentration The nominal concentration refers to the intended or specified concentration used in an experimental setting of a substance in a solution, i.e. the total amount of substance divided by the volume of exposure medium to which the substance is added (e.g. mol/L medium; Groothuis et al., 2015).

Reverse Dosimetry Reverse dosimetry refers to the process of predicting or estimating an external dose level of a substance based on given internal exposure information (concentration, e.g. in blood/plasma or target tissues or organs) applying PBK models. In the context of the quantitative interpretation of in vitro toxicity data, a link between the in vitro exposure and the internal concentration is made, and related reverse dosimetry provides insight into external dose levels related to bioactivity measured in vitro. In the context of human biomonitoring data, reverse dosimetry refers to the process of determining the amount of a substance a person is expected to have been exposed to, based on concentrations determined in human samples, such as blood, urine or tissue.

Reference point A dose derived from an experimental/observational dose–response relationship (e.g. BMDL/NOAEL/LOAEL) that reflects the critical toxicological effect. This can also be read as point of departure (PoD). In this Scientific Opinion, reference point is also used in some cases when not necessarily referring to the critical toxicological effect.

Unbound concentration	The unbound concentration of a test chemical in exposure medium or in a biological matrix (e.g. plasma or tissue), also called free concentration or freely available concentration. The unbound concentration can be lower than the nominal concentration, due to substance binding to medium constituents or plastics, non-specific cell binding and/or evaporation.

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PANEL MEMBERS

Tamara Coja, Pauline Adriaanse, Judy Choi, Antonio Finizio, Maeva Giraudo, Thomas Kuhl, Emily McVey, Francesca Metruccio, Martin Paparella, Silvia Pieper, Eugenio Scanziani, Ivana Teodorovic, Paul Van der Brink, Martin Wilks.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Monitoring of pesticide residues and metabolites in food commodities and human samples

In this Appendix, background information is provided on monitoring of pesticide residues and metabolites in food commodities and human samples.

Regulatory frameworks and monitoring requirements

The regulation and monitoring of pesticide residues in food are critical for ensuring consumer safety and compliance with legal standards across the European Union (EU). While food monitoring is strictly regulated under EU law, human biomonitoring of pesticide residues is not mandated by specific EU regulations but remains important for assessing internal exposure and research initiatives. Various legislative frameworks and analytical methodologies establish guidelines for pesticide residue analysis in food commodities and human biomonitoring studies.

The approval and monitoring of pesticide active substances in the EU are subject to several regulatory frameworks. Regulation (EC) No. 1107/2009 sets out the rules for approving active substances used in plant protection products, while Commission Regulation (EU) No. 284/2013 outlines the data requirements for active substances, as specified in Article 8(1)(c) of Regulation (EC) No. 1107/2009. Section 5 of Part A of this regulation provides guidelines for analytical methods necessary for both pre-authorisation data generation and post-authorisation control and monitoring purposes.

Regulation (EC) No 396/2005 establishes the obligation of Member States to conduct controls to ensure that food placed on the market complies with established maximum residue levels (MRLs). Two primary control mechanisms are in place: the EU-coordinated multiannual control programme (EU MACP) and the multiannual national control programmes (MANCP). The EU MACP requires all Member States to monitor a harmonised list of food products and pesticide residues, with sampling efforts proportionate to national populations (EFSA, 2015). The MANCP allows individual Member States to focus on specific food products suspected of exceeding MRL or pose potential risks to consumer safety. As of 14 December 2022, Article 30 of Regulation (EC) No. 396/2005 was replaced by Article 155 of Regulation (EU) No. 2017/625, with further amendments introduced by Regulation (EU) No. 2021/1355, effective from 15 December 2022, reinforcing the requirement for Member States to establish and maintain national control programmes in accordance with the updated provisions.

Human biomonitoring of pesticide residues: methodological and regulatory considerations

Human biomonitoring (HBM) is crucial for assessing internal exposure to chemicals, including pesticide residues, through the analysis of biological samples such as blood, urine, hair and, in some cases, other matrices. HBM data are meaningful to assess population-level exposure, enabling comparison of actual in vivo exposure against reference points, including those extrapolated from DNT IVB data using PBK modelling-facilitated QIVIVE. While there is no specific EU regulation mandating Member States to conduct biomonitoring studies of pesticide residues, various initiatives have been developed to harmonise approaches across countries. For example, EU-funded projects, including HBM4EU and PARC, have developed harmonised methodologies for sample collection, chemical analysis and data interpretation for the biomonitoring of a broad range of chemicals, including pesticides.

Sample collection involves gathering human samples such as blood, urine or hair, and less commonly, other invasive samples (e.g. tissues, cerebrospinal fluid) that will be analysed for pesticides and metabolites. For chemicals with short half-lives (typically less than 1 day), careful consideration of the timing of sample collection and relevance of the selected biomarker of exposure are critical to ensure reproducibility and meaningful interpretation of results.

Each matrix poses distinct analytical challenges and requires specific sample preparation techniques such as homogenisation, filtration and dilution. These steps are necessary to preserve analyte integrity, reduce matrix interferences and concentrate the analytes of interest during extraction, ensuring accurate and reliable analysis.

Quantitative analysis of pesticides and their metabolites in human samples requires robust extraction methods, sophisticated instrumental analysis and stringent quality control measures to generate accurate and reliable data. The analytical workflow, which typically includes sample collection, preparation, extraction and instrumental analysis (EC, 2023), must be designed to generate high-quality data. Such data provide direct evidence of internal exposure, which may ultimately support both risk assessment and regulatory decision-making.

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APPENDIX B

In vivo internal exposure metrics and in vitro exposure metrics for QIVIVE

In this Appendix, more information is provided on in vivo internal exposure metrics and in vitro exposure metrics for QIVIVE, supporting the recommended approach as described in Section 4 in the Scientific Opinion.

In vivo internal exposure metrics in QIVIVE

As stated in Section 4, there is currently no general consensus on the internal exposure metric to link in vitro (effect) concentrations to in QIVIVE. Many QIVIVE studies relate in vitro concentrations to unbound maximum or steady-state concentrations in plasma. Notably, in the first published QIVIVE example, neurotoxic dose levels were predicted based on effect concentrations from SHSY5Y cells; here, the nominal in vitro concentration was linked to the total brain concentration described by the PBK model (Dejongh et al., 1999). In a second study, developmental toxicity dose levels were predicted based on effect concentrations from ES-D3 cells, where the unbound concentration in vitro was linked to the unbound plasma concentration in vivo (Verwei et al., 2006). In the high throughput QIVIVE approaches published by the US-EPA (e.g. Rotroff et al., 2010; Wetmore et al., 2012), nominal in vitro concentrations were linked to total (bound and unbound) plasma concentrations. Other QIVIVE studies have linked cell-associated concentrations in vitro to total target tissue concentration in the PBK models (e.g. Fragki et al., 2023; Mielke et al., 2017).

It is generally assumed that the unbound concentration at the site of action is related to the toxicological effect of the substance (Pelkonen et al., 2008). Depending on the mode of action, the molecular target at the site of action can be an extracellular target (e.g. membrane receptor), a target in the cytoplasm or in the nucleus (e.g. cytosolic/nuclear receptor) or a target in an organelle (e.g. mitochondrial respiratory chain complex). For highly permeable chemicals interacting with plasma membrane receptors, the unbound concentration at the site of action is assumed equal to the blood or plasma unbound chemical concentration (Chu et al., 2013; Guo et al., 2018). This assumption may not be valid when the molecular target is intra- or subcellular, especially for chemicals for which transporter proteins at the tissue-blood barrier cause 'asymmetry' between the tissue and blood unbound chemical concentrations (Chu et al., 2013; Guo et al., 2018).

For both these extra- and intracellular molecular targets, the unbound chemical concentration in the interstitial fluid surrounding the cells can be considered as the relevant exposure metric in vivo. Generally, most (low-tier) PBK models do not describe concentrations in the interstitial fluid and the unbound concentration in plasma is considered a relevant surrogate. Using the unbound plasma concentration for the QIVIVE would assume that the unbound plasma concentration is (at equilibrium) equal to the unbound concentration in the interstitial fluid. This is a reasonable assumption but may lead to an overestimation of unbound interstitial concentrations of various chemicals in the brain, for which the blood–brain-barrier may limit the transfer of chemicals to the brain tissue (Pardridge, 2005). Assuming the unbound plasma concentration to be the same as the unbound brain concentration (in absence of transporter-mediated distribution) is supported by a study of Kalvass and Maurer (2002), who showed that for a set of pharmaceuticals for which no active transport was involved in the blood–brain-barrier, the brain to plasma concentration ratio was well predicted by the ratio fraction unbound in plasma/fraction unbound in brain tissue.[26] The brain to plasma concentration ratio was not predicted by the ratio fraction unbound in plasma/fraction unbound in brain tissue for pharmaceuticals for which active transport was expected in the blood–brain-barrier, and the brain to plasma concentration ratio was generally overpredicted. Therefore, in absence of kinetic data to make a robust estimation of the ratio of brain to plasma concentrations, use of the unbound plasma or blood concentration can be considered an adequate conservative estimate of unbound concentrations in the brain.

In vitro exposure metrics for QIVIVE

As stated in Section 4, an understanding of the in vitro distribution kinetics of test chemicals in the DNT IVB is essential for robust QIVIVE as it provides insight into how the nominal concentration relates to the biologically effective dose (BED, Escher et al., 2011). Chemicals added to exposure medium (i.e. the nominal concentration) can bind to lipid and protein constituents in exposure medium, evaporate and bind to plastics, reducing the fraction that is available for interacting with cells (Groothuis et al., 2015). If the molecular target of the chemical is an extracellular receptor, the unbound concentration in the medium may be considered the most relevant in vitro exposure metric. In the absence of transporter-mediated cell and tissue distribution, this in vitro exposure metric is also relevant for chemicals that have a cytosolic or nuclear receptor as their molecular target, assuming that the unbound concentration outside the cell is (at equilibrium) equal to the unbound concentration inside the cell (Pelkonen et al., 2008).

[26]Support for assumption that unbound plasma concentration is the same as the unbound brain concentration:

– fu, plasma = unbound concentration plasma/total concentration plasma.

– fu, brain = unbound concentration brain/total concentration brain.

– fu, plasma/fu, brain = unbound concentration plasma/total concentration plasma × total concentration brain/unbound concentration brain.

– in case unbound concentration plasma = unbound concentration brain: fu, plasma/fu, brain = total concentration brain/total concentration plasma.

In the absence of information on the unbound concentration in vitro, QIVIVE based on the linking of the nominal concentration in vitro to the total plasma concentration in vivo can be considered to provide a conservative estimate of the OEDs as the protein concentration in in vitro culture media is generally lower than the protein concentration in blood or plasma with a typically higher unbound concentration in vitro compared to the unbound concentration in blood or plasma (Henneberger et al., 2021; Lin et al., 2025; Nicol et al., 2024; Punt et al., 2021). However, one should be cautious using this approach for volatile and unstable chemicals that may evaporate and degrade, respectively, when unbound, which make in vitro testing difficult, resulting in a high degree of uncertainty regarding the actual in vitro exposure. The (effective) concentrations in vitro may also be lower than expected due to binding of chemicals to plasticware used in the dosing of the in vitro systems (e.g. pipette tips, dilution series vials) or the plastic plates themselves (Groothuis et al., 2019). As evaporation and degradation are dynamic processes, standard mass balance models are poorly suited to detect such issues. It is also noted that mass balance models do not consider plasticware before the 'entrance' of a chemical in the in vitro system (like pipets, medium dilution Eppendorf/centrifuge tubes). For non-volatile, stable chemicals with minimal binding to plastic, and limited sorption to cells and/or medium constituents, unbound concentrations are expected to be similar to nominal concentrations. For such chemicals, a high unbound plasma fraction is also expected in vivo and linking the nominal in vitro concentration to the total in vivo concentration would be close to linking the unbound in vitro and unbound in vivo concentration, thus providing a relevant starting point for the QIVIVE.

When assuming the intracellular unbound concentration (at equilibrium) to equal to the unbound concentration outside the cell (in the cell culture medium in vitro and in the interstitial fluid in vivo), one must consider that in certain cases the equilibrium may get disturbed, e.g. in case of 'pH trapping' of weak acids and bases when the internal pH and external pH differ. Non-specifically cytotoxic or baseline toxicants, whose main action is considered the accumulation into and disruption of cell membrane function, the steady-state (equilibrium) concentration in the biological membrane better explains the variability in effect between chemicals in vitro and is considered a better exposure metric for QIVIVE than the intracellular or extracellular unbound concentration (Lee et al., 2022; Mielke et al., 2019; Nunes et al., 2023). For lipophilic chemicals, the unbound concentration is a negligibly small part of the fraction bound to the membrane, and thus the more easily discernible cell-associated concentration, i.e. the total cellular concentration, approximates the membrane-bound concentration and may be used as in vitro exposure metric for QIVIVE instead (Groothuis et al., 2015; Lee et al., 2022; Mielke et al., 2019; Nunes et al., 2023). QIVIVE based on total cell concentration in vitro was shown to provide better estimations of ibuprofen liver toxicity than when based on the unbound concentration in the medium (Mielke et al., 2017, 2019). So far, limited examples are available that performed QIVIVE based on total cell concentration (e.g, Fragki et al., 2023; Mielke et al., 2017). When assessing reactive mechanisms in vitro, like for rotenone and paraquat, an unbound or membrane-bound steady-state or equilibrium in vitro cannot be assumed and additional parameters (e.g. reaction rate with the target site and regeneration of the target site) are needed for characterising the in vitro biologically effective dose for QIVIVE (Escher et al., 2011; Groothuis et al., 2015; Gülden et al., 2010).

For the assessment of in vitro distribution kinetics (i.e. the extent to which the chemical distributes to cells, well plate plastic and headspace) of test chemicals in the DNT IVB, the following properties[27] of each in vitro system should be considered and defined over the exposure time as they influence the difference between the nominal and biologically effective concentration in vitro (Groothuis et al., 2015; Proença et al., 2021):

1. Cell culture setup (e.g. spheroids, adherent monolayers)
2. Cell density
3. Cell volume
4. Cell doubling time
5. Cell growth
6. Cell lipid content
7. Cell protein content
8. Biotransformation enzyme activity
9. Xenobiotic transporter activity
10. Well plate plastic type
11. Well dimensions
12. Presence, type, concentration and volume of extracellular matrix
13. Presence and type of sealer
14. Basal exposure medium type, pH
15. Exposure medium volume
16. Exposure medium temperature
17. Exposure medium supplements (e.g. fetal bovine serum, FBS, B12 supplement, etc.)
18. Exposure regimen (e.g. single vs. repeat dosing, frequency of medium replacement)
19. Exposure time

[27]Related information also available in GIVIMP (OECD, 2018).

The highest test concentration tested for assessing the in vitro distribution kinetics of the test chemical in the test systems should not cause more than 10% cytotoxicity (OECD, 2018).

The following chemical properties should be considered and defined as they, in addition to the assay properties, help define the difference between the nominal and biologically effective dose in vitro:

1. Molecular weight
2. pKa
3. Log P, log D7.4 and/or log lipid/water partition coefficients
4. Potency
5. Stability in medium
6. Metabolic pathway and intrinsic clearance in vitro
7. Transporter affinity
8. Henry's law constant
9. Plasma protein binding
10. Solubility
11. Isomerism
12. Mechanism of toxicity (e.g. molecular initiating event in vitro)

Knowledge of chemical and assay characteristics help parameterise in vitro distribution kinetic models described in Section 4 to estimate the difference between the nominal, unbound medium, cell-associated, cell bound and unbound intracellular concentration, as well as design extraction experiments to analytically assess medium and cell-associated concentrations in time (Groothuis et al., 2019; Kramer et al., 2012; Nunes et al., 2023; Wilmes et al., 2013). Available analytically assessed in vitro kinetic behaviour of chemicals over time data circumvent the uncertainties in in vitro distribution kinetics by in silico model estimates, especially for volatile, instable, biotransformed and/or transporter substrates, to determine the C_{max} for QIVIVE (Nunes et al., 2023) or, in the case of reactive chemicals, the AUC (Escher et al., 2011; Lee et al., 2022) for QIVIVE. Indications on how to carry out the cytotoxicity test are given in the OECD GIVIMP document (OECD, 2018) and in the Scientific Opinion on comparative in vitro metabolism studies (EFSA PPR Panel, 2021). The total incubation time depends on the aim of the test, the biological test system used and its configuration, as described above. In addition, the number of time points (at least 3) and sampling schedule should be enough to provide reliable fitting of concentration-time profiles in vitro (Mielke et al., 2017; Truisi et al., 2015).

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APPENDIX C

Considerations for oral equivalent doses (OEDs) obtained with Tier 1 PBK models

In this Appendix, considerations are provided whether QIVIVE approaches using Tier 1 PBK models generally provide relevant and conservative estimations of QIVIVE-based OEDs for DNT for the non-breastfed infant and the developing embryo/fetus, considering QIVIVE based on unbound concentrations in medium and QIVIVE based on cell-associated concentrations.

Non-breastfed infant

QIVIVE based on the unbound medium concentration

In the absence of active transport, it can be assumed for most chemicals that the unbound concentration outside the cell is, at equilibrium, the same as the unbound concentration inside the cell (Pelkonen et al., 2008). However, this may not be true for chemicals with low passive permeability. Also, specific pH trapping mechanisms can cause different unbound concentrations in cells and in plasma. In case the unbound concentration is assumed to be the same as the unbound concentration inside the cell, the unbound chemical concentration in the interstitial fluid surrounding the cells can be considered as the relevant exposure metric in vivo. As low-tier PBK models generally do not describe concentrations in the interstitial fluid, the unbound plasma concentration is used for the QIVIVE based on the assumption of equilibrium between plasma and interstitial fluid. This is expected to lead to an overestimation of unbound interstitial concentrations in the brain as the blood–brain-barrier (BBB) may limit the entry of chemicals into the brain (Pardridge, 2005).

The relevance of considering the unbound concentration in plasma as surrogate for unbound concentration in the brain is supported by studies that showed that for pharmaceuticals not actively transported through the BBB, the brain to plasma concentration ratio was well predicted by the ratio between the fraction unbound in plasma and the fraction unbound in brain tissue, suggesting that for such chemicals the unbound fraction in the brain is in equilibrium with the unbound fraction in plasma. For pharmaceuticals, of which active transport was expected, this assumption may, however, lead to overestimation of brain concentrations (Kalvass & Maurer, 2002; Maurer et al., 2005).

Altogether, for a Tier 1 PBK model, linking the unbound concentration in vitro to the unbound concentration in blood/plasma is considered a conservative and adequate approach for QIVIVE in this exposure scenario.

QIVIVE based on the cell-associated concentration

Predictions of tissue concentrations in PBK models are typically driven by tissue:plasma partition coefficients that follow principles of perfusion limitation. Various in silico tools are available that allow the prediction of these coefficients based on chemical characteristics like Log *P*, pKa and fraction unbound in plasma. When the chemical of interest is within the applicability domain of the tool, these in silico predictions are generally considered relevant. However, these tools generally do not consider active transport processes and/or barriers like the BBB. Therefore, predicted brain concentrations based on brain:plasma partition coefficients may be overestimated for chemicals for which the BBB might limit their translocation from plasma to interstitial fluid. Therefore, linking the cell-associated concentration in vitro to the total brain concentration in the PBK model is considered to provide a conservative estimate of the OED for this exposure scenario and is suitable to use in low-tier PBK modelling. If in vivo data are available on brain to plasma concentration ratios in juveniles, these can be used for PBK model parameterisation to more accurately describe brain exposure. As the PBK model complexity does not necessarily need to be increased, this can still be considered Tier 1 modelling.

Developing embryo/fetus

QIVIVE based on the unbound medium concentration

In the absence of active transport, the unbound concentration in the maternal plasma in the placenta can be assumed to be the same as the unbound concentration in the fetal plasma under equilibrium (Balhara et al., 2022). While unbound concentrations may be the same, total concentrations may differ as protein concentrations (such as albumin) available for chemical binding in maternal and fetal blood may differ, which also change during pregnancy (Krauer et al., 1984). Chemicals that are substrates of certain transporter proteins, such as P-glycoprotein (PgP) and breast cancer resistance protein (BCRP), have limited transport across the placenta as shown in animal studies comparing fetal exposure in wild-type and specific transporter protein knockout mice (Chen et al., 2023) and in human perfused placenta studies (Pollex et al., 2008). No studies have yet reported a net flux of a chemical to the fetus. Therefore, the unbound maternal plasma concentration is a relevant and conservative surrogate for the fetal unbound plasma concentration.

As the relevant target for the QIVIVE of DNT IVB data is the developing brain, embryonic/fetal brain exposure is to be considered, i.e. further disposition of the chemical from fetal plasma to developing brain tissue and interstitial fluid. There is limited knowledge on the translocation of chemicals from the fetal circulation to the developing brain. However, the BBB becomes functional immediately after its formation to provide essential protection during early embryonic development

(Abbott et al., 2010). This early functionality is reflected by the expression of tight junction proteins, such as occludin and claudin-5, in the developing human brain as early as 12 weeks of gestation (Virgintino et al., 2000). These proteins are essential for sealing the spaces between endothelial cells, regulating paracellular permeability and maintaining the structural integrity of the BBB. Also, members of the ATP-binding cassette family of transporter proteins, expressed in the endothelial cells of the BBB have been reported to play important roles in protecting the brain against chemical exposure (Abbott et al., 2010). The expression of these transporters is also present in fetal stages (Eng et al., 2022), but differences in expression and possible age-related variations may affect the extent of protection (Koehn et al., 2019). For a conservative estimate, it can be assumed that the fetal BBB is not active, and consequently, the unbound chemical concentration in the interstitial fluid surrounding the brain cells is the same as the unbound concentration in the maternal plasma, which is used as a surrogate for the unbound concentration in fetal plasma.

The use of maternal plasma concentration as a relevant internal exposure metric to link in vitro toxicity data to embryonic/fetal toxicity in vivo is supported by the publication of Daston et al. (2010), who published an 'exposure-based validation list for developmental toxicity screening assays' based on maternal plasma C_{max} values for positive and negative chemicals. Furthermore, proof-of-principle examples in the literature that compared QIVIVE-based external equivalent doses for developmental toxicity based on linking of the (unbound) maternal plasma concentration to the (unbound) concentration in vitro showed to be in general in the same order of magnitude as reference points (NOAELs/BMDLs) derived from animal studies (Louisse et al., 2010; Louisse et al., 2015; Strikwold et al., 2013; Wang et al., 2021). It is noted that QIVIVE cases for DNT have been reported in the literature (Algharably et al., 2023; Dobreniecki et al., 2022; Johansson et al., 2024; Maass et al., 2023), but limited information is available how the obtained OEDs from the QIVIVE relate to reference points derived from animal studies.

In a study that reviewed paired maternal and cord blood data, it was reported that for most chemicals, cord blood concentrations were lower than maternal blood concentrations (Aylward et al., 2014). Higher cord blood concentrations were seen for some brominated flame retardants, polyaromatic hydrocarbons and metals.

To provide more insight whether the maternal unbound plasma concentration is a conservative estimate for the fetal unbound plasma concentration, a targeted literature search was performed (see for details on the search and the related data extracted Annex C).

The analysis of the in vivo kinetic data obtained from that literature search shows that for most of the chemical data collected, fetal plasma concentrations were lower than maternal plasma concentrations in animal studies (Figure A1A). Human data obtained from paired umbilical cord plasma and maternal plasma samples in toxicokinetic studies (Figure A1B) support the results from Aylward et al. (2014), i.e. that concentrations in umbilical cord blood are generally lower than in maternal blood. For more details on the underlying data, see Annex C.

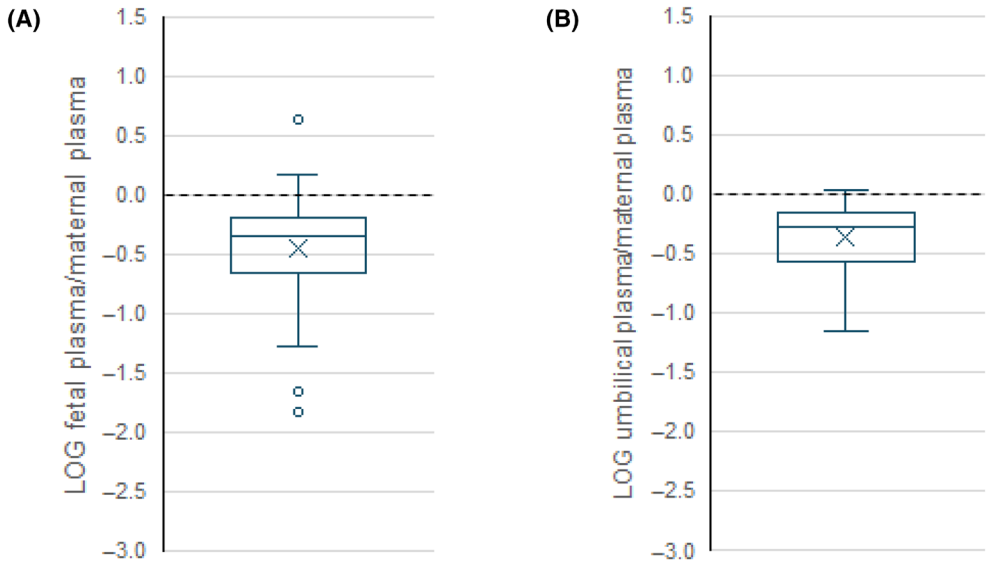

FIGURE A1 Ratios of fetal to maternal plasma exposure from toxicokinetic studies in animals (A) and ratios of umbilical to maternal plasma exposure from human toxicokinetic studies (B). Data include ratios of either reported AUC, C_{max} or concentration at given time after exposure (paired samples). Different exposure routes are included and data are presented for parent chemicals and metabolite(s) if quantified as well. Box plots are presented for log-transformed data, amounting to 47 data points for (A) and 29 datapoints for (B). The X represents the mean of each dataset. The dashed line represents a ratio of 1 (fetal concentration = maternal concentration). Boxplots are created in Excel (see Annex C). No reliability assessment has been performed for the studies collected. See for details on the literature search and data extraction Annex C.

In the earlier stages of pregnancy, the placenta is not yet functional and exposure to the embryo is not dependent on placental transfer. Estimation of embryonic concentrations may be cumbersome, but it is considered that the application of maternal unbound plasma concentrations will also provide sufficiently conservative OEDs for the embryonic stages.

Altogether, linking the unbound concentration in the in vitro culture medium to the unbound concentration in maternal plasma in a low-tier PBK model provides a relevant and conservative estimate of the external equivalent dose for this exposure scenario, and is, therefore, adequate to be used as first tier in PBK modelling.

QIVIVE based on cell-associated concentration

The estimation of fetal brain concentrations in higher tier PBK modelling is considered highly uncertain when no in vivo kinetic data are available on paired samples on maternal plasma and fetal (brain) levels. Therefore, as a pragmatic approach, when cell-associated in vitro concentrations are to be linked to total tissue concentrations in Tier 1 PBK modelling for exposure scenario 1, total tissue concentration in fetal brain can be estimated as a worst case (and therefore considered adequate for Tier 1 modelling) by applying the estimated brain:plasma partition coefficient to the fetal plasma concentration. As explained above, the unbound concentration in fetal plasma is equal to (at equilibrium) the unbound concentration in maternal plasma. Total fetal plasma concentrations may be slightly different than total maternal plasma concentration related to different levels of binding proteins (Krauer et al., 1984). In the absence of data to correct for such differences, no correction is needed as these differences are expected to be limited.

An in vivo kinetic study on fetal exposure in relation to maternal internal exposure for the DNT chemical methylmercury (MeHg) is available (Sakamoto et al., 2018). In this study, maternal and fetal blood concentrations were similar, while fetal brain concentrations were slightly higher than maternal brain concentrations but around tenfold lower than fetal and maternal blood concentrations. This would indicate that QIVIVE based on linking the free in vitro concentration to free maternal plasma concentration would provide an adequate OED with a Tier 1 PBK model, whereas a QIVIVE based on linking the cell-associated concentration to the total brain concentration may provide a conservative OED. If the data in Sakamoto et al. (2018) are considered reliable for maternal plasma and fetal brain concentrations, going to higher tier modelling may not be needed in a QIVIVE case for MeHg, in case these data could be applied to adjust the brain:plasma partition coefficient in a Tier 1 model.

The literature search performed and described in detail in Annex C provided data on paired internal exposure information in mother (maternal plasma or maternal brain) and fetus (either fetal plasma, fetal brain and/or total fetus). Data on fetal plasma:maternal plasma ratios are presented in Figure A1. Data on fetal brain:maternal plasma ratio, whole fetus:maternal plasma ratio and fetal brain:maternal brain ratio are presented in Figure A2A–C, respectively. These data show that for more than 50% of the chemicals for which data were collected, the maternal plasma concentration is lower than the fetal brain concentration (Figure A2A) or whole fetus concentration (Figure A2B). On the other hand, for more than half of the chemicals reviewed, fetal brain concentrations were higher than maternal brain concentrations (Figure A2C).

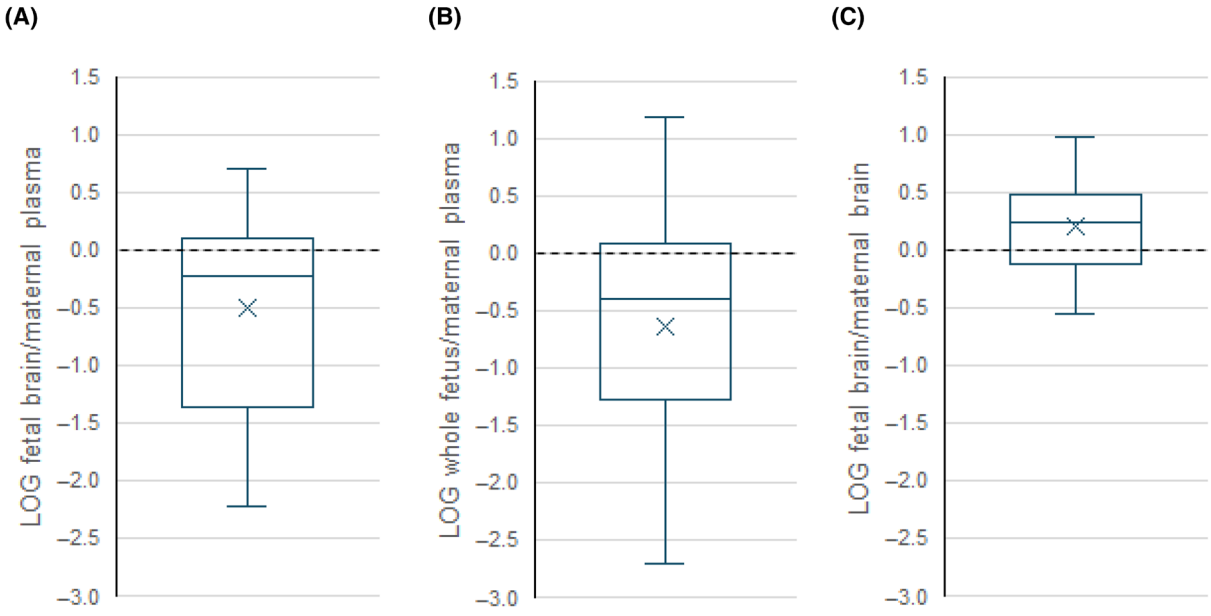

FIGURE A2 Ratios of fetal brain to maternal plasma exposure (A), whole fetus to maternal plasma exposure (B), fetal brain to maternal brain exposure (C). Data include ratios based on AUC, C_{max}, or concentration at certain time after exposure (paired samples) from animal studies applying different exposure routes. Data are presented for parent chemicals and metabolite(s) if quantified as well. Box plots are presented for log-transformed data, amounting to 18 data points for (A), 247 data points for (B) and 14 data points for (C). The X represents the mean of each dataset. The dashed line represents a ratio of 1. Boxplots are created in Excel (see Annex C). No reliability assessment has been performed for the studies collected. See for details on the literature search and data extraction Annex C.

Altogether, linking the cell-associated concentration to the surrogate fetal brain concentration, estimated by applying the brain:plasma partition coefficient to the maternal plasma concentration, provides a relevant and conservative estimate of the OED for this exposure scenario and is therefore adequate to be used as Tier 1 approach.

As explained in the modelling strategy described in Section 5.5, if the QIVIVE-based OED(s) based on the Tier 1 PBK modelling do not allow to conclude that available toxicological reference values (TRVs) are sufficiently protective, higher tier PBK modelling can be considered. Such higher tier modelling requires an extensive kinetic dataset to allow adequate parameterisation of PBK models.

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ANNEX A
Protocol for a PPR Panel Scientific Opinion on the application of PBK modelling for the QIVIVE of DNT IVB data for pesticide active substances.

ANNEX B
List of available PBK model guidance documents.

ANNEX C
Kinetic data extracted from literature studies on paired internal exposure mother versus fetus.

ANNEX D
Overview of pesticide active substances that have harmonised classification for 'may cause harm to breast-fed children' (H362).

ANNEX E
Overview of in vivo, in vitro and/or in silico-based approaches that may support chemical-specific parameterisation of PBK models.

ANNEX F
Reporting template QIVIVE DNT IVB data.

ANNEX G
Public consultation on the draft Scientific Opinion.