**WEIGHT OF EVIDENCE/UNCERTAINTY IN HAZARD ASSESSMENT**

**Background Document & Examples**

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# **Foreword**

The aim of this background document and the accompanying template is to assist to:

* harmonise the use of Weight of Evidence (WoE) and uncertainty assessment within ECHA processes
* improve transparency in regulatory decision making relevant for REACH, CLP and BPR purposes
* facilitate the use and integration of alternative methods and all available information in hazard assessment

It contains:

* An introduction and definitions in the field of WoE and uncertainty approaches
* An explanation of the relevance of use of WoE and uncertainty assessment for ECHA processes
* The basics of the methodologies for WoE and uncertainty assessment
* Two examples using the template for WoE/Uncertainty

The document does not deal explicitly with how to assess the WoE analysis but rather what elements need to be considered within the building of WoE approaches and the assessment of uncertainty.

The background document and the accompanying template are not formal ECHA Guidance Documents.

The document focuses on hazard assessment, although the principles described would be applicable for exposure and risk assessment where weighing of evidence applies.

# **Introduction**

For the purpose of this document a review of existing work in the field of Weight of Evidence and uncertainty by other regulatory agencies, international organisations and recent publications has been performed. The material used is listed in the Reference Section of this document.

## **Weight of Evidence Approach**

There is no formal adopted definition of the Weight of Evidence (WoE) approach.

A number of available descriptions of WoE approach from ECHA Guidance Document, WHO/IPCS publications, SCENHIR, EFSA, US OSHA (see Reference Section of this document) have been considered.

In line with these descriptive definitions available it is recommended that the WoE approach can be described as follows:

*Weight of Evidence approach can be generally described as a stepwise process/approach of collecting evidence, assessing, integrating and weighing them to reach a conclusion on a particular problem formulation with (pre)defined degree of confidence.*

The following proposed 6 steps are considered to constitute the backbone of the WoE approach:

1. **Problem formulation**
2. **Collection & Documentation of all information**
3. **Assessment of quality of individual evidence (reliability, relevance, adequacy)**
4. **Integration & Weighing of Evidence**
5. **Application of levels of confidence**
6. **Uncertainty Analysis**
7. **Conclusion**

The stepwise approach proposed above is in line with ECHA Guidance on IR/CSA (R2, R3, R4, R5) where the following steps are described: collection, evaluation, adaptation and generation of information.

**Figure 1** provides an overview of the proposed iterative WoE approach indicating also the conceptual difference between “WoE approach” versus “WoE analysis” with the latter being only part of the WoE approach, i.e. not constituting the whole WoE approach (note that this distinction this is not always clear within the ECHA Guidance Documents).

The principles of the WoE approach as recommended in this document intend to facilitate structured and transparent way of performing and presenting hazard assessment (hazard identification, hazard characterisation). Following the steps of the WoE approach prompts the assessor to identify the needs and gaps in the hazard assessment and facilitates regulatory dialogue and decision making.

The weighing of evidence occurs at the **step of Integration & Weighing of Evidence** (Step 4). However, it is noted that partial weighing of evidence occurs firstly at the **step of assessment of quality of individual evidence** (Step 3) since metrics/criteria such as quality, adequacy and relevance are used in weighing the evidence for the purpose of the assessment. These feed into the next step of integration and weighing of evidence (Step 4), where the evidence is weighed collectively using other types of metrics such as consistency and plausibility (see Section 9).

For the evaluation and integration of all available information, ECHA Guidance (IR/CSA R.4) describes it as case-dependent. “*It is influenced by the relation between the amount of information needed and the importance of the decision to be taken and also by the likelihood of, and, consequences for, the decision based on that information being wrong. It is important to document and communicate how the evidence based approach was used in a reliable, robust and transparent manner.”*

**The WoE approach/process is iterative**: all steps are interconnected and changes might be needed if for example it is recognised in a later stage that some evidence initially given less weight or even disregarded should be taken into account more prominently or *vice versa*.

1. **Uncertainty Analysis**
2. **Collection and Documentation of all information**
3. **Assessment of quality of individual evidence**
4. **Integration and Weighing of Evidence (WoE analysis)**
5. **Application of levels of Confidence**
6. **Conclusions**
7. **Problem formulation**

**Figure 1:** Overview of iterative Weight of Evidence approach

## **Terms used in the Weight of Evidence area**

The following terms are often used in the field of WoE and may be part of WoE approach but are not equivalent to the complete WoE approach itself as described in this document and proposed in the corresponding template.

The following list of terms aims to highlight the variety of terms encountered within the WoE area. The recommended terms are those indicated within the Template for WoE/Uncertainty.

Some of these terms refer to **criteria**, which are applied when constructing the WoE approach and/or evaluating certain of its elements whereas others refer to specific **steps** of the WoE approach. The terms mentioned in this section are encountered either in Guidance documents or within hazard assessments.

Some definitions of terms used as criteria for WoE construction/evaluation are available within ECHA Guidance R2-4 whereas some terms are general descriptive terms.

* **Quality of data** (assessment of adequacy, reliability, relevance) (according to ECHA Guidance IR/CSA R.4)
* **Adequacy**: defining the usefulness of data for hazard/risk assessment purposes. Where there is more than one study for each endpoint, the greatest weight is attached to the studies that are the most relevant and reliable. For each endpoint, robust summaries need to be prepared for the key studies.
* **Reliability**: evaluating the inherent quality of a test report or publication relating to preferably standardised methodology and the way the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings. Reliability of data is closely linked to the reliability of the test method used to generate the data.
* **Relevance**: covering the extent to which data and tests are appropriate for a particular hazard identification or risk characterisation.
* **Adequacy for purpose:** “*available information should be evaluated in relation to the level of certainty and accuracy needed to meet the regulatory requirement…it should be considered whether generation of new data would impact such regulatory decision making.”*
* **Completeness:** “*conclusion on comparison between available evidence and evidence required for a specific purpose (e.g. information requirement under REACH)”*
* **Coverage of key parameters:** assessment of parameters examined within an experimental study and comparison with the parameter requirements of a standard (test) protocol or an information requirement under REACH. It can be seen as a measure of relevance, reliability and adequacy (quality of data) but is useful to be considered separately. No formal explanation is provided in ECHA Guidance IR/CSA R.4
* **Consistency:** (see Section 4 of the Template WoE/Uncertainty)
* **Specificity:** (see Section 4 of the Template WoE/Uncertainty)
* **Plausibility/Likelihood:** (see Section 4 of the Template WoE/Uncertainty)
* **Expert Judgment:** General descriptive term for use of expert scientific knowledge where precise evidence is not available
* **Expert judgment elicitation:** General descriptive term
* **Strength of Evidence** (see Section 2.5 of this document)
* **Confidence** (see Section 2.5 of this document)
* *Terms that are part of the WoE approach but not the approach itself*
* Weight of Evidence analysis
* Overall weight of evidence
* *Additional terms:*
* Weight of Evidence flag (used in OECH Harmonised Templates/IUCLID study records to indicate if evidence is used in a WoE approach)
* Evidence: Information that is relevant for assessing the answer to a specified question (according to EFSA Guidance on WoE). This is a general term that can be any type and piece of information that can be used in the WoE approach (experimental data from *in vivo* or *in vitro* assays, information from similar chemicals, QSARs, monitoring data)
* Systematic Reviews: Systematic reviews as developed in the field of evidence based medicine include similar steps to the WoE approach (collection of information, assessment, and interpretation). An overview of the Systematic Review approach is provided by OHAT (OHAT, 2015) and EFSA (EFSA, 2010., EFSA, 2015a)

* WoE adaptations: General description of use of multiple lines of evidence for omitting of testing, which is further elaborated in ECHA Guidance (IR/CSA R.4). According to ECHA Guidance IR/CSA: *a Weight of evidence approach mentioned in Annex XI Section 1.2 of REACH, integrates available information from guideline tests, non-guideline tests and other types of information which may justify adaptation of the standard testing regime*.

Further elaboration of terminology in the field of WoE and uncertainty is available within the ECHA Guidance (IR/CSA R.2-R.6, R.19), the CLP Guidance as well as within the *EFSA Guidance on Uncertainty in Scientific Assessments* and the *EFSA Guidance on the use of Weight of Evidence Approach in Scientific Assessments* (EFSA, 2017).

## **Uncertainty Definitions**

Definitions of uncertainty vary depending on the context and the regulatory framework. In general uncertainty is defined as “*lack of knowledge*” or “*limitations in knowledge*”.

* Uncertainty can be defined as “*imperfect knowledge concerning the present or future state of an organism, system or (sub) population under consideration*” (WHO/IPCS 2014)
* According to *EFSA Guidance on Uncertainty in Scientific Assessments*: “*uncertainty refers to all types of limitations in the knowledge available to assessors at the time an assessment is conducted and within the time and resources agreed for the assessmen*t”.

Uncertainty informs on confidence during the WoE approach.

It should be noted that uncertainty analysis is a process that runs through any type of assessment (applicable for both hazard and exposure components) and can vary in terms of complexity depending on the type of problem formulation.

The process can be qualitative, semi-quantitative or quantitative depending on the methodology used to reach a conclusion on uncertainty. For cases requiring detailed uncertainty analysis more complex statistical approaches are used to derive conclusions (ECHA Guidance IR/CSA, R.19), in contrast to simple qualitative conclusions such as “*high confidence*”, “*low uncertainty*”.

## **Relationship of WoE approach and uncertainty analysis**

Similar to the WHO/IPCS *Guidance on Uncertainty in Hazard assessment* (WHO/IPCS 2014), **Figure 2** below shows how uncertainty analysis and WoE approach run in parallel during hazard assessment (hazard identification and/or hazard characterisation/ dose response relationship) and that both processes share as common components the derivation of confidence levels and conclusions. Both WoE and uncertainty analysis feed in hazard assessment components.

Problem Formulation

Weight of Evidence Approach

Confidence Levels/Remaining Uncertainty

Uncertainty Analysis

Conclusion/Recommendations

Hazard Assessment

**Figure 2:** Interrelationship of uncertainty analysis and Weight of Evidence feeding in Hazard Assessment.

Uncertainty analysis, like the WoE approach, is also a process that includes steps that correspond to the steps described in the WoE approach in this document:

* problem formulation
* identification and description of uncertainties
* assessment of individual sources of uncertainty
* assessment of overall impact of all identified uncertainties

This stepwise approach for uncertainty analysis is further elaborated within the *WHO/IPCS Guidance on uncertainty in hazard assessment* (WHO/IPCS 2014), and is similar to the *ECHA Guidance on uncertainty analysis* in ECHA IR/CSA R.19*.*

Although from both WoE approach and the uncertainty analysis, confidence levels and remaining uncertainty can be derived/recorded, it is noted that the uncertainty analysis provides tools/methods that can also be used within the WoE to quantify uncertainty elements. This may be the case for example in hazard characterisation, for assessing remaining uncertainty in the derivation of threshold levels (e.g. DNELs). Although the uncertainty analysis can use higher tier methods (statistical approaches) to derive quantitative confidence levels if required by the problem formulation, in ‘regular’ hazard assessments, the qualitative approach of deriving confidence levels (high, medium or low) is seen as more pragmatic.

## **Application of Weight of Evidence/uncertainty analysis for ECHA processes**

The WoE approach and uncertainty analysis are relevant for all REACH/CLP/BPR processes dealing with hazard assessment (hazard identification and/or hazard characterisation and/or dose-response characterisation:

* REACH Dossier Evaluation
* REACH Substance Evaluation
* CLH process
* REACH Restrictions/Authorisations/SVHC identification
* Biocidal active substances Assessment
* REACH Socio-Economic analysis

The registrants or dossier submitters are expected to prepare and present the WoE approach (all steps), whereas ECHA (e.g. during CCH) and its Committees are expected to perform the examination of the assessment elements of the WoE evidence approach and the conclusion steps (Figure 3).

**ECHA/Committees**

**Registrant/Applicant/ Dossier Submitter**

1. **Uncertainty Analysis**
2. **Collection and Documentation of all information**
3. **Assessment of quality of individual evidence**
4. **Integration & Weighing of Evidence (WoE analysis)**
5. **Application of levels of Confidence**
6. **Conclusions**
7. **Problem formulation**

**Figure 3:** The WoE approach within ECHA processes

The degree of application of the WoE and uncertainty analysis approaches depends on the process and is case dependent.

## **Current approaches of Weight of Evidence/uncertainty in ECHA processes**

The WoE approach is described within ECHA Guidance (IR/CSA R.2-4), the CLP and BPR Guidance Documents and the Information Toolkit. Uncertainty analysis is described in ECHA Guidance IR/CSA R.19.

For some of the steps of the WoE approach (as defined in the steps proposed in earlier sections of this document) no harmonised templates are available; e.g. for the integration and weighing of evidence part, for recording collection of data and search strategy. Consequently this leads to some limitations regarding the application of the complete WoE approach within ECHA processes (Registration, Dossier & Substance Evaluation, CLH, SVHC Identification, PBT Assessment, Endocrine Disruption Identification etc.).

In particular, in relation to Figure 1:

1. General **problem formulation** is often well defined per REACH/CLP/BPR process. However more prescriptive problem formulation is not used to allow interlink with the weighing of evidence step.
2. The **collection and documentation of all information** for all REACH/CLP/BPR processes is not always transparently recorded in IUCLID section 13 by the registrant or Dossier Submissions by the eMSCAs or ECHA opinions from Committees.
3. The **assessment of the quality of individual evidence** is performed using the Klimisch criteria in all REACH/BPR processes for the reliability of the evidence. For adequacy and relevance the elements for assessment are in ECHA Guidance IR/CSA R.4.

Transparent documentation of evidence is guided by the use of the OECD structured harmonised templates (OHTs) within IUCLID and predefined formats of the CSR report and other process related reports.

However, for CLP processes (CLH), the Klimisch criteria are not often used in the decision making process for the scoring of studies in terms of reliability. The CLP Guidance is not explicit on the use such criteria and allows more flexibility.

As per ECHA Guidance IR/CSA R.4, “*the scoring of information should not exclude all unreliable data from further consideration by expert judgment because of possible pertinence of these data related to the evaluated endpoints*.”

1. There is no specific section in ECHA formats of opinions (or in IUCLID) to address the **integration and weighing of the evidence**.

For the integration of all evidence and subsequent weighing no formal template is used and is most often presented in a narrative format; in specific processes like CLH or SVHC identification the elements that are required for the WoE analysis step are most often addressed although not always using the same terminology.

1. **Application of level of confidence and uncertainty analysis** are not usually presented in a structured format. These are usually presented not as separate components but mixed with the narrative format of other steps of the WoE approach. Quantitative approaches for uncertainty analysis (more relevant for hazard characterisation approaches) are not often used. With the exception of the Restrictions and the SVHC identification templates/formats, there is no specific section in ECHA formats of opinions to address uncertainty explicitly (either in the form of confidence levels or in the form of formal uncertainty analysis).

Specific elements of uncertainty in hazard characterisation, as for example uncertainty in inter-species extrapolation for DNEL/AEL derivation in hazard characterisation, are normally dealt with by application of standard assessment factors. Additional recommendations for dealing with uncertainty in such type of assessment is provided in ECHA Guidance Documents for the use of Physiologically based Pharmacokinetic (PBPK) modelling (IR/CSA R.8), Chemical specific adjustment factors (CSAFs) (IR/CSA R.8) and the respective WHO/IPCS Guidance Documents on CSAFs, PBPK, and uncertainty.

# **Methodologies for Weight of Evidence approach/uncertainty**

Tiered approaches are used in hazard assessments with lower tiers consisting of hazard screening or assessment with defaults approaches and higher tiers employing more advanced methodologies for hazard assessment. Different tools/methodologies can be used within the WoE approach and uncertainty analysis with higher uncertainty/lower confidence being accepted for lower tiers of assessment (e.g. priority setting) compared to higher tier applications (establishment of protection goals).

Depending on the problem formulation (e.g. for a specific regulatory application such as PBT assessment, endocrine disruption identification, endpoint hazard assessment) different tools can be used to weigh the evidence, assess the uncertainty and conclude on the adequacy for purpose from all available information (see next sections for details).

It should be noted that in certain applications, uncertainty analysis might be needed as full separate process; for example defining threshold levels (DNELs, AELs) in hazard characterisation might need to define percentage of covered populations. In such cases complete uncertainty analysis for this particular part of hazard characterisation can be an additional component to the WoE approach.

Further information on uncertainty analysis in hazard assessment is available in the ECHA Guidance IR/CSA R.19, the *WHO/IPCS Guidance on Uncertainty in hazard characterisation* (WHO/IPCS 2014) the *EFSA Guidance document on uncertainty* (see list of References).

The following sections provide an overview of how each part of the proposed WoE approach as shown in figure 1 of this document can be interpreted/filled in, in addition to the recommendations provided within the Template for WoE/Uncertainty.

## **2.1 Problem Formulation**

Problem formulation, as described within WHO/IPCS publications, defines the scope and goals in relation to the assessment, the level of uncertainty that is acceptable as well as the urgency of the assessment.

In practice, problem formulations that ECHA will encounter correspond to the processes under REACH, CLP, and BPR. The actors involved can vary being either the registrant, a CA submitting a dossier, ECHA evaluating a dossier etc.

*Examples of problem formulation* within REACH/CLP/BPR processes are:

Hazard assessment related:

* PBT assessment: does the substance fulfil the PBT criteria?
* SVHC/ED: Is the substance an endocrine disrupter meeting Art. 57(f)??
* C&L: is there sufficient scientific evidence that the mechanism/ mode of action or end-point specific (adverse) effect is not relevant to humans?
* REACH/BPR/CLP: Is there sufficient evidence to conclude on the presence or absence hazardous properties for a specific endpoint?
* REACH/BPR: Adaptation of information requirements according to Annex XI (REACH)/ Annex IV (BPR) provisions (e.g. adaptation based on Weight of evidence approach, adaptation based on Read-across approach etc.). Is the registrants WoE adaptation adequate?

Risk assessment/impact related:

* Assessment of authorisation applications: risk/benefit analysis, e.g. do the benefits outweigh the identified risks?

Problem formulation can be phrased either as general question in relation to a specific process or can have additional sub-questions that would assist the assessor to perform WoE/uncertainty analysis (for example with specific questions on the elaboration on Mode of action (MoA) analysis and human relevance).

The process of WoE and uncertainty analysis are iterative processes linking the conclusion with the problem formulation and answering the question “Do we know enough to characterise a physio-chemical, (eco)toxicological property of a substance?”. The same applies for risk assessment related aspects (hazard + exposure). The level of uncertainty/confidence that will be acceptable depends on the goals set within the problem formulation.

## **2.2 Collection and documentation of all information**

**Documentation of search strategy & documentation/reporting of evidence**

ECHA Guidance (IR/CSA R2-R6) provides a list of sources to be used for the collection of relevant information/evidence in regulatory hazard assessment.

The search strategy should be reported, as well as all evidence collected.

IUCLID Section 13 should be used for the documentation by registrants and dossier submitters. A summary of the information collected can be provided as part of the WoE approach within the assessment report.

## **2.3 Assessment of quality of individual evidence**

For regulatory processes (REACH, BPR) the ECHA Guidance IR/CSA R.4 provides the elements for the assessment of the quality of data (on the basis of reliability, relevance and adequacy). The **Klimisch criteria** are proposed for the assessment of reliability of experimental studies. For evidence that do not fulfil Klimisch criteria, the assessment is considered within the frame of the integration of evidence regarding consistency/ specificity rather than dismissing the evidence as non-relevant beforehand.

For other type of evidence (e.g. QSAR, use of read-across, non-standard *in vitro* assays) ECHA Guidance provides criteria for their assessment (ECHA Guidance IR/CSA R.4 & R.6).

For the assessment of relevance and adequacy ECHA Guidance IR/CSA R.4 and the Template for WoE/uncertainty provide the elements that need to be considered.

Additional examples are given here below regarding the assessment of the quality of evidence (mostly in relation to reliability) but do not represent a complete list:

* The Grading of Recommendations Assessment, Development and Evaluation approach (GRADE) for systematic assessment of the quality, strength and certainty of evidence (GRADE, 2013) provides an approach for assessing overall quality of data within the area of clinical data.
* Systematic review frameworks (e.g. EFSA 2010, EFSA 2015) provide also principles of elements to take into account for assessment of quality of evidence and some more specific for systematic reviews of endocrine specific chemicals (Vandenberg et al, 2016).
* The OHAT approach (Office Health Assessment and Translation) (OHAT) for Systematic Review and Evidence Integration (OHAT, 2015) provides an approach for assessing study quality, or “risk of bias.” The tool applies a parallel approach to the evaluation of risk of bias for human and experimental animal studies.
* The SCENHIR opinion (SCENHIR, 2012) on WoE approach and uncertainty analysis provides recommendations regarding the assessment and weighing of individual evidence similar to the principles described within ECHA Guidance Documents (R.2-R.4).
* The US OSHA (OSHA, 2012) Guidance described the assessment of individual data similar to the principles within ECHA Guidance documents (CLP, REACH and Biocides).
* The TOXRTOOL: The software-based tool “ToxRTool” (**Tox**icological data **R**eliability Assessment **Tool**) was developed within the context of an ECVAM funded project to provide comprehensive criteria and guidance for evaluations of the inherent quality of toxicological data, thus making the decision process of assigning reliability categories more transparent and more harmonised (<https://eurl-ecvam.jrc.ec.europa.eu/about-ecvam/archive-publications/toxrtool>)
* Criteria of the Science in Risk Assessment and Policy (SciRAP) tool [www.scirap.org](http://www.scirap.org): **SciRAP** (Science in Risk Assessment and Policy) is a web-based reporting and evaluation resource developed to facilitate and increase the use of peer-reviewed toxicity and ecotoxicity literature in regulatory risk assessment of chemicals. It can be used for assessing reliability of *in vivo* data.
* The CRED-system, (Moermond et al. 2016).

## **2.4 Integration and weighing of evidence (WoE analysis)**

Depending on the problem formulation different methodologies can be used to integrate evidence and perform WoE analysis.

Methodologies vary in terms of complexity and range from general schemas/frameworks consisting of set of questions to more elaborate methodologies.

It is noted that a framework as such should not be confused with the problem formulation. For instance, an adaptation of information requirements with the use of read-across is a problem formulation whereas the WoE analysis can be used to evaluate the applicability of read-across by a set of questions within a read across framework.

Within a specific framework, another framework may be needed depending on process/type of work (e.g. CLP categorisation schemes can be considered as classification frameworks with WoE approaches which can further benefit from MoA approach inclusion).

The choice of the methodology used for integration of evidence is interrelated to the communication needs, goals and degree of confidence required set within the problem formulation.

For specific problem formulations, like in case of assessment of endocrine disruption assessment, the OECD conceptual framework for testing and assessment of endocrine disrupters provides guidance on the assessment of the different lines of evidence for this particular type of assessment (<http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm> ).

Some systematic Review Frameworks (as mentioned in Section 2.3 of this document) also provide approaches for integration of evidence in addition to methods for assessing the quality of evidence.

Gross et al (Gross et al, 2017), also provide an overview of methods for integration of evidence.

The tables below provide an overview of the frameworks/tools that can be considered either as a WoE approach on their own or may include WoE approach type of considerations.

The elements recommended to be considered for the integration and weighing of evidence (consistency, specificity, likelihood/biological plausibility, temporality) are further elaborated in the Template for WoE/Uncertainty and are largely based on the Bradford Hill considerations as used within the WHO/IPCS Mode of Action framework (WHO/IPCS, 2013).

**Table 1:** List of criteria or rules within regulatory setting (REACH, BPR, CLP) that define the use/application of WoE approach, the degree of analysis and guide through a series of questions for regulatory decision making

|  |  |  |
| --- | --- | --- |
| **Regulatory criteria /rules in relation to WoE approach** | **REACH/CLP/BPR process relevance** | **Description** |
| ***PBT Criteria*** | * Registration * PBT assessment * Dossier Evaluation * Substance Evaluation * BPR * SVHC/ Restrictions/ Authorisations | Framework for regulatory decision making  Quantitative assessment of PBTs  Specific to PBT assessment  Set of criteria |
| ***Draft ED criteria for Biocides*** | * BPR | <http://ec.europa.eu/health/endocrine_disruptors/policy/index_en.htm>  List of criteria containing most elements of the WoE approach steps presented in this document |
| ***CLP Criteria*** | * Registration * Dossier Evaluation * Substance Evaluation * CLH * BPR * SVHC/ Restrictions/ Authorisations | Framework for regulatory decision making  Endpoint specific  Specific to CLP  Set of criteria  Quantitative for some endpoints and qualitative for some other |
| ***Exclusion criteria and comparative assessment under BPR (Article 5, BPR)*** | * BPR | List of criteria for the approval of biocidal active substances |
| ***REACH and Biocides specific and general rules for adaptation of information requirements*** | * Registration * Dossier Evaluation * Substance Evaluation * BPR | Considerations that can be used to weigh the available evidence for regulatory decision making processes  Specific rules providing conditions regarding standard testing, further testing and options for omitting the standard test  Annex XI of REACH and Annex IV of BPR “adaptation of information requirements” provide a set of conditions (testing scientifically unnecessary) that can use conceptually a WoE approach (consideration of existing data, read-across etc.) |
| ***ITS (Integrated Testing Strategies) per endpoint as described for many endpoints in REACH/CLP/BPR*** | * Registration * Dossier Evaluation * Substance Evaluation * CLH * BPR | Set of questions to reach conclusion on a specific hazardous property and/or need for further information  Can assist in deciding which evidence to consider and how to weigh it  Depending of endpoint complexity and specificity of questions vary |

**Table 2:** Methods and/or frameworks which are considered to apply a complete WoE approach or a partial WoE approach

|  |  |  |
| --- | --- | --- |
| **Methods for WoE approach and/or analysis/integration of evidence** | **REACH/CLP/BPR process relevance** | **Description** |
| ***WHO/ IPCS Mode of Action/WoE Framework (WH0/IPCS 2013)*** | * Registration * Dossier Evaluation * Substance Evaluation * CLH * BPR * SVHC/ Restrictions/ Authorisations | Qualitative and/or quantitative assessment of WoE (including human relevance analysis)  Can be used for both HH and ENV assessments  Allows detailed integration of evidence and WoE analysis  It is a stepwise WoE approach on its own similar to the one presented in this document  Can be used with varying availability of evidence (from poor to data rich chemicals) |
| ***OECD Adverse Outcome Pathways (AOPs)*** *(see References OECD AOP)* | * Registration * Dossier Evaluation * Substance Evaluation * CLH * BPR * SVHC/ Restrictions/ Authorisations | Similar to WHO/IPCS MoA Framework above  AOPs/MoA can also be used within an IATA or standalone |
| ***OECD IATAs*** *(see References OECD IATA)* | * Registration * Dossier Evaluation * Substance Evaluation * CLH * BPR * SVHC/ Restrictions/ Authorisations | Overall framework to integrate all sources of evidence (read-across, *in vivo*, *in vitro*)  Similar concept to the stepwise WoE approach presented in this document  Similar templates to the WoE integration templates in ECHA Guidance for acute toxicity may be used  Can be used with or without AOP/MoA templates  If AOP/MoA framework analysis performed those templates from AOP/MoA area are used |
| ***OECD Endocrine Disruption Conceptual framework*** *(see References)* | * Registration * Dossier Evaluation * Substance Evaluation * CLH * BPR * SVHC/ Restrictions/ Authorisations | Framework that provides guidance on assessment of different lines of evidence for endocrine disruption assessment |

**Table 3:** List of other methodologies that can be seen following similar principles or complementing the WoE approach (for one or more steps of the WoE approach)

|  |  |  |
| --- | --- | --- |
| **REACH/CLP/BPR** | **REACH/CLP/BPR** | **Description** |
| ***ECHA Read-across framework (ECHA, 2017)*** | * Registration * Dossier Evaluation * Substance Evaluation * CLH | Qualitative assessment of read-across plausibility as part of evaluation of adaptation of information requirements  Similar principles as the ones within the WoE approach (assessment of individual information and integration and weighing of evidence between structurally similar chemicals) |
| ***Uncertainty methodologies*** *(WHO/IPCS 2014, ECHA Guidance IR/CSA R.19)* | * Registration * Dossier Evaluation * Substance Evaluation * CLH * BPR * SVHC/ Restrictions/ Authorisations | Qualitative  (Semi) quantitative approaches  Used in uncertainty analysis and/or in WoE approach within the WoE analysis step |
| ***Systematic Review Frameworks*** *(see Section 2.3)* | * Registration * Dossier Evaluation * Substance Evaluation * CLH * BPR * SVHC/ Restrictions/ Authorisations | Similar to the stepwise Weight of Evidence approach  Emphasis on collection of lines of evidence in a systematic way  Consideration of assessment and integration of all lines of evidence |

Some methodologies that are used to refine hazard assessment could contribute to the WoE analysis with the aim to reduce uncertainty. Such methods are for example:

* PBPK (physiologically based pharmacokinetic) modelling for refining NOAEL derivation or derivation of assessment factors
* Chemical Specific Adjustment factors (CSAFs)

## **2.5 Application of levels of confidence**

Confidence levels/strength of evidence can be assigned either to individual evidence and/or to the overall evidence assessment. In order to perform the weighing of evidence within the step of integration of all evidence, confidence levels need to be transparently recorded.

Confidence levels are derived taking into account the outcome of the weighing of the evidence (both individually and collectively) using the metrics/criteria specified in the corresponding steps of the WoE approach (such as adequacy, relevance, reliability for individual evidence assessment, and consistency/specificity, plausibility/likelihood, temporality for WoE analysis).

Confidence levels are usually expressed as:

* High (strong)
* Medium (moderate)
* Low (weak)

The confidence levels for each line of evidence should feed to the judgement of the overall confidence level that take into account all the evidence in an integrated and weighed mode.

For each line of evidence confidence levels can have as underlying documentation:

* Qualitative elements (e.g. plausibility)
* Semi-quantitative elements (e.g. temporality)

Level of confidence of each line of evidence is derived by combining the quality assessment elements of each line of evidence (relevance, adequacy, reliability) with the consistency, plausibility and temporality elements.

In order to judge which overall confidence level to assign when concluding the WoE approach according to the SCENIHR opinion on WoE/Uncertainty the following criteria are proposed for defining confidence levels:

**Strong**: Coherent evidence from human and one or more other lines of evidence (in particular mode/mechanistic studies) in the absence of conflicting evidence from one of the other lines of evidence (no important data gaps)

**Moderate**: good evidence from a primary line of evidence but evidence from other lines of evidence is missing (important data gaps)

**Weak**: weak evidence from the primary lines of evidence (severe data gaps)

**Uncertain**: due to conflicting information from different lines of evidence that cannot be explained in scientific terms

**Weighing of evidence not possible**: No suitable evidence available

The elaboration of confidence levels, and their underlying documentation, would in general depend on the needs defined by each process and problem formulation.

Similarly the OECD AOP programme for the assessment of AOPs provides general guidance for characterizing the level of quantitative understanding of a key events relationships for an adverse outcome pathway as weak, moderate, or strong (Annex 2 of the “*Users' Handbook supplement to the Guidance Document for developing and assessing Adverse Outcome Pathways*", OECD Series on Adverse Outcome Pathways, No. 1, OECD Publishing, Paris, available at: <http://dx.doi.org/10.1787/5jlv1m9d1g32-en> )

## **2.6 Uncertainty Analysis**

The principles of uncertainty analysis and its relevance within the WoE approach are described in Section 1.4 of this document.

Elaboration of uncertainty as a result of the analysis of the evidence and their integration is interrelated to the overall confidence levels derived from the previous steps of the WoE approach.

On a case-by-case basis, it might be appropriate to record uncertainties in a separate table to address the source of the uncertainty and the impact to the assessment.

However, it might also be sufficient to elaborate on the uncertainty within the step of derivation of levels of confidence.

## **2.6 Conclusions**

When concluding in a WoE approach and/or uncertainty analysis process, depending on the problem formulation and the need to go to a higher tier assessment (usually when confidence levels are below the goal set in problem formulation), a number of approaches can be used to increase confidence levels:

* Gather further information
* More elaborate methodologies could be incorporated in the analysis

e.g., PBPK modelling, CSAFs, MoA analysis, statistical approaches to define remaining uncertainty in quantifiable manner – probabilistic approaches.

# **References**

[Ågerstrand M](https://www.ncbi.nlm.nih.gov/pubmed/?term=%C3%85gerstrand%20M%5BAuthor%5D&cauthor=true&cauthor_uid=26682868), [Beronius A](https://www.ncbi.nlm.nih.gov/pubmed/?term=Beronius%20A%5BAuthor%5D&cauthor=true&cauthor_uid=26682868)., Weight of Evidence evaluation and systematic review in EU chemical risk assessment: Foundation is laid but guidance is needed. 2016, Environ Int. 92-93: 590-6

ANSES: Évaluation du poids des preuves à l’Anses : revue critique de la littérature et recommandations à l’étape d’identification des dangers. 2016, <https://www.anses.fr/fr/system/files/AUTRE2015SA0089Ra.pdf>

ECHA Guidance Documents and Practical Guides: <http://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment>

ECHA Guidance R.2-R.6 Overall framework for meeting the information requirements on intrinsic properties of substances (Chapter R.2), guidance on collection of available information (Chapter R.3), evaluation of information (Chapter R.4)

ECHA Guidance R.8 Determination of Derived No-Effect-Levels (DNEL) (or other qualitative or semi-quantitative measures of potency of the substance)

ECHA Guidance R.19 Uncertainty Analysis

ECHA RAAF 2017, Read-across assessment framework, available at: <https://echa.europa.eu/documents/10162/13628/raaf_en.pdf/614e5d61-891d-4154-8a47-87efebd1851a>

ECHA Guidance on the application of the CLP criteria <https://echa.europa.eu/guidance-documents/guidance-on-clp>

EFSA, 2010, Guidance for those carrying out systematic reviews. Application of systematic review methodology to food and feed safety assessments to support decision making. EFSA Journal 2010; 8(6):1637, doi:[10.2903/j.efsa.2010.1637](http://dx.doi.org/10.2903/j.efsa.2010.1637), <http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2010.1637/epdf>

EFSA Guidance on Uncertainty in EFSA Scientific Assessments, Draft: <http://www.efsa.europa.eu/sites/default/files/consultation/150618.pdf>

EFSA, 2015a. Scientific report on Principles and process for dealing with data and evidence in scientific assessments. EFSA Journal 2015; 13(5):4121, 35 pp. doi:[10.2903/j.efsa.2015.4121](http://dx.doi.org/10.2903/j.efsa.2015.4121)”

EFSA, 2015b, Workshop on Increasing Robustness, Transparency and Openness of Scientific Assessments. Workshop report available at: <http://www.efsa.europa.eu/en/supporting/pub/913e>

EFSA, 2017, Guidance on the use of the weight of evidence approach in scientific assessments, 3 August 2017, <http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2017.4971/full>

GRADE, 2013, The Grading of Recommendations Assessment, Development and Evaluation approach (GRADE) for systematic assessment of the quality, strength and certainty of evidence: The GRADE Working Group. (2013). GRADE handbook for grading quality of evidence and strength of recommendations. (O. A. Schünemann H, Brożek J, Guyatt G, Ed.). Retrieved from <http://gdt.guidelinedevelopment.org/central_prod/_design/client/handbook/handbook.html>

Gross M., Green M. R., Weltje L., Wheeler R. J., Weight of Evidence approaches for the identification of endocrine disrupting properties of chemicals: Review and recommendations for EU regulatory application., Regulatory Toxicology and Pharmacology, 91 (2017) 20-28.

Moermond, C. T. A., Kase, R., Korkaric, M., & Agerstrandk, M. (2016). CRED: CRITERIA FOR REPORTING AND EVALUATING ECOTOXICITY DATA. Environmental Toxicology and Chemistry, 35(5), 1297-1309. doi:10.1002/etc.3259

OECD AOP, Information and Guidance documents on AOPs available at: <http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm>

OECD IATA, IATA Guidance available at: <http://www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm>

OECD 2016a, Guidance document on the reporting of defined approaches to be used within integrated approaches to testing and assessment: <http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282016%2928&doclanguage=en>

OECD 2016b, Guidance document on the reporting of defined approaches and individual information sources to be used within integrated approaches to testing and assessment (IATA) for skin sensitisation: <http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282016%2929&doclanguage=en>.

OECD Conceptual Framework for testing and assessment of endocrine disrupters available via: <http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm>

OHAT, 2015: Handbook for conducting a literature-based health assessment using OHAT approach for systematic review and evidence integrati: <https://ntp.niehs.nih.gov/ntp/ohat/pubs/handbookjan2015_508.pdf>

Rhomberg LR, Hypothesis-based weight of evidence: an approach to assessing causation and its application to regulatory toxicology., 2015, Risk Anal. 35, 1114-24.

SCENIHR, 2012, Memorandum on the use of the scientific literature for human health risk assessment purposes, weighing of evidence and expression of uncertainty; available at: <http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_s_001.pdf>

US OSHA, 2012, Guidance on Data Evaluation for Weight of Evidence Determination <https://www.osha.gov/weightofevidence/woe_guidance.pdf>

Vandenberg, L. N., Ågerstrand, M., Beronius, A., Beausoleil, C., Bergman, Å., Bero, L. A., … Welshons, W. (2016). A proposed framework for the systematic review and integrated assessment (SYRINA) of endocrine disrupting chemicals. Environmental Health, 15(1), 74. <http://doi.org/10.1186/s12940-016-0156-6>

WHO/IPCS 2013, M. E. Meek, A. Boobis, I. Cote, V. Dellarco, G. Fotakis, S. Munn, J. Seed and C. Vickers. Article first published online: 25 October 2013 | DOI: 10.1002/jat.2949 <http://onlinelibrary.wiley.com/doi/10.1002/jat.v34.1/issuetoc> <http://www.who.int/ipcs/methods/harmonization/areas/cancer/en/>

WHO/IPCS 2010, Guidance on principles of characterising and applying PBPK models in risk assessment <http://www.who.int/ipcs/methods/harmonization/areas/pbpk/en/>

WHO/IPCS 2005, Chemical specific adjustment factors for interspecies differences and human variability: Guidance document for use of data in dose/concentration-response assessment <http://www.who.int/ipcs/methods/harmonization/areas/csaf/en/>

WHO/IPCS 2014, Guidance on Evaluating and Expressing Uncertainty in Hazard Assessment is available in Harmonization Project Document No. 11 <http://www.who.int/ipcs/methods/harmonization/areas/hazard_assessment/en/>

# **Appendix**

## **Example of use of the Weight of Evidence/Uncertainty Template for environmental hazard assessment**

**Foreword**

The following example is only illustrative for a case of evaluation of Persistence of a substance (5-sec-butyl-2-(2,4-dimethylcyclohex-3-en-1-yl)-5-methyl-1,3-dioxane [1], 5-sec-butyl-2-(4,6-dimethylcyclohex-3-en-1-yl)-5-methyl-1,3-dioxane [2] [covering any of the individual stereoisomers of [1] and [2] or any combination thereof])

that has been assessed as Substance of Very High Concern because of its vPvB properties by the Member State Committee.

The data set and its assessment was taken from the Support Document for SVHC identification available at: <https://echa.europa.eu/documents/10162/ebe9403b-13e3-47da-b5a8-e83286f2fc30>

Modifications were made and the example is only presented to illustrate the use of the proposed template and its tables and does not necessarily reflect the actual assessment of **this** substance for **vP**.

1. **Problem Formulation**

A weight-of-evidence determination according to the provisions of Annex XIII of REACH is used to identify if the substance meets the criteria for vP.

1. **Collection and Documentation of all information**

Information/evidence used in the approach include:

* Experimental studies from Endpoint study records from corresponding IUCLID Registration Dossiers
* QSAR Results

**Abiotic Degradation/Hydrolysis**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Source Name** | **Date of search** | **Type of evidence** | **Link/Reference** | **Reason for exclusion from WoE approach** |
| QSAR Toolbox v3.3 Hydrolysis simulator | 02/06/2014 | Estimated /predicted | <https://www.qsartoolbox.org/> | N/A |
| OECD TG 111 Key study | 02/06/2014 | Experimental Study | ECHA Dissemination website | N/A |
| OECD TG 111 Supporting study | 02/06/2014 | Experimental Study | ECHA Dissemination website | N/A |

**Biodegradation in water**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Source Name** | **Date of search** | **Type of evidence** | **Link/Reference** | **Reason for exclusion from WoE approach** |
| EPIWIN | 02/06/2014 | Estimated /predicted | <https://www.epa.gov/tsca-screening-tools/download-epi-suitetm-estimation-program-interface-v411> | N/A |
| CATABOL | 02/06/2014 | Estimated /predicted | <http://oasis-lmc.org/products/models/environmental-fate-and-ecotoxicity/catabol-301c.aspx> | N/A |
| OECD QSAR Toolbox | 02/06/2014 | Estimated /predicted | <https://www.qsartoolbox.org/> | N/A |
| METEOR | 02/06/2014 | Estimated /predicted | <https://www.lhasalimited.org/products/meteor-nexus.htm> | N/A |
| Biodegradation Screening test OECD TG 301B | 02/06/2014 | Experimental Study | ECHA Dissemination website | N/A |
| Biodegradation Screening test OECD TG 301F | 02/06/2014 | Experimental Study | ECHA Dissemination website | N/A |
| Biodegradation Screening test | 02/06/2014 | Experimental Study | ECHA Dissemination website | N/A |
| Manometric Respirometry Study OECD TG 302C | 02/06/2014 | Experimental Study | ECHA Dissemination website | N/A |
| Aerobic mineralisation study OECD TG 309 | 02/06/2014 | Experimental Study | ECHA Dissemination website | N/A |

1. **Assessment of quality of individual evidence**

The evidence used in the assessment of persistence are examined regarding their quality by using the ECHA Guidance R.4.

For the experimental studies available, the Klimisch scoring is used for assessment of reliability.

For the estimated properties (QSAR results) the principles for evaluation of estimated results generated with QSAR approaches as described within ECHA Guidance has been followed. The QMRF/QPRF for each model are not presented in this example.

In addition, it is considered more appropriate to evaluate the quality of these evidence within the integration and weighing of evidence, using plausibility and consistency as measures of quality.

**3.1 Abiotic Degradation / Hydrolysis**

|  |  |  |  |
| --- | --- | --- | --- |
| **Type of Evidence / Data Source - Reference** | **Relevance** | **Reliability** | **Adequacy** |
| OECD TG 111 Key study | Study appropriate for investigation of hydrolysis as function of pH | Klimisch score 2 | Adequate on basis of high reliability and high relevance |
| OECD TG 111 Supporting study | Relevant protocol but critical parameters influencing evaluation not considered adequately | Klimisch score 3 | Adequacy to be considered within integration of evidence |

The hydrolysis simulator of the OECD QSAR toolbox (v3.3) shows the possibility of acid catalysed hydrolysis, without providing any indication of a rate of hydrolysis. The predicted mechanism of hydrolysis is identical for both positional isomers with undefined stereochemistry. Therefore, the figures below depict only one of the two positional isomers. As shown in Figure 1, hydrolysis would start with the opening of the 1,3-dioxane ring and yields, after cleavage of the ring and subsequent dehydrogenation, hydrolysis product 4, which is a pre-registered substance known as 2,4-dimethylcyclohex-3-ene-1-carbaldehyde (CAS number 68039-49-6). Hydrolysis under neutral or basic conditions is not predicted by the hydrolysis simulator. Thus, based on these QSAR estimates hydrolysis of the substance seems plausible at low pH values, but not likely under environmentally relevant pH values.

Two GLP-compliant studies are available that investigated hydrolysis of the substance as a function of pH according to OECD TG 111. Both studies were conducted with nonradiolabelled substance. The registrant reported for the key study a half-life of 738.9 hours at pH 4 and 25 °C ], and in the supporting study a half-life of > 275 < 830 hours at pH 4 and 25 °C .

The supporting study investigated the hydrolysis of non-radiolabelled substance in buffered aqueous solutions of pH 4, 7 and 9 at 50 and 55°C for up to 5 days. Hydrolysis at pH 4 was additionally also investigated at 65°C for up to 42 hours. In all tests, a trend of time related decrease of the substance was observed. For pH 7 and pH 9, a DT50 could not be calculated due to irregularities of the log (relative) concentration versus time curves. For pH 4, the registrant estimated a DT50 at 25 °C of 275-830 hours following extrapolation. However, inspection of the chromatograms by the evaluating MSCA showed that the peak corresponding to hydrolysis product 4 (see Figure 1) was absent in all samples. As non-radio-labelled material was used, a mass-balance could not be obtained, and dissipation by e.g. volatilisation cannot be ruled out. Considering theabove remarks, this study is considered unreliable by the eMSCA, and is assigned a Klimisch score of 3.

The key study also investigated hydrolysis of non-radiolabelled substance in buffered aqueous solutions of pH 4, 7 and 9, but the test setup was more extensive as for each pH tests were conducted at 50, 60 and 70 °C for up to 11 days. Furthermore, this study also investigated hydrolysis in 0.22 μm filtered Rhône water (pH 8.2) that was collected upstream of Givaudan activity, at 50 °C for 11 days. In a non-GLP part of the study, hydrolysis products were identified for a single sample (pH 4, 60 °C, 144 h). Hydrolysis product 4 and another highly similar hydrolysis product were detected. Quantification of hydrolysis products (hydrolysis products 3 and 4 from Figure 1, as well as the reduction/oxidation products of hydrolysis product 4 shown in Figure 2) was conducted for the test at pH 4 and 70 °C. Only hydrolysis product 4 could be detected. Concentration of hydrolysis product 4 increased limitedly with time, while the concentration of the substance decreased rapidly especially at the start of the test. This shows that while hydrolysis under acid conditions occurs, other processes, such as binding to the test vessel and/or volatilization, also contributed to the reduced substance levels in the aqueous phase. Consequently, hydrolysis of the substance was most likely overestimated.

The registrant reported for the substance a DT50 of 738.9h at pH 4 following extrapolation to 25 °C. The evaluating MSCA reassessed the data and calculated a DT50 of 1830h at pH 4 following extrapolation to 12 °C. This shows that even at acidic conditions hydrolysis of the substance is very slow at an EU relevant environmental temperature. For pH 7 and 9, DT50 values could not be extrapolated to 12 °C, due to the irregularities of the log (relative) concentration versus time curves. For river water with pH 8.2, a DT50 of 241 h was calculated at 50 °C, demonstrating that at 50 °C dissipation in the hydrolysis test occurs almost a factor two slower at pH 8.2 compared to pH 4.

The key study strongly indicates that hydrolysis half-life at environmentally relevant pH and temperature will be considerably longer than 1830 hours. Thus, even though this study was not conducted with radiolabelled material, which most likely overestimated hydrolysis of the substance, it has been demonstrated that hydrolysis of the substance is at most very limited at relevant environmental conditions. The results from this study are considered reliable with restrictions, and are assigned a Klimisch score of 2.

**3.2 Biodegradation in water**

**Experimental data: Screenings tests & Simulation tests (water and sediment)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Type of Evidence / Data Source - Reference** | **Relevance** | **Reliability** | **Adequacy** |
| Biodegradation Screening test OECD TG 301B | Yes, according to test guideline protocol covering parameters required for assessment. | Klimisch score 2 | Adequate study for biodegradation in water |
| Biodegradation Screening test OECD TG 301F | Yes, according to test guideline protocol covering parameters required for assessment. | Klimisch score 1 | Adequate study for biodegradation in water |
| Biodegradation Screening test OECD TG 301 | Yes, according to test guideline protocol covering parameters required for assessment. | Klimisch score 1 | Adequate study for biodegradation in water |
| Manometric Respirometry Study OECD TG 302C | Yes, according to test guideline protocol covering parameters required for assessment. | Klimisch score 1 | Adequate study for biodegradation in water |
| Aerobic mineralisation study OECD TG 309 | Yes, according to test guideline protocol covering parameters required for assessment. | Klimisch score 2 | Adequate study for biodegradation in water |

There are four biodegradation screening studies available for the substance, of which three are available on the public dissemination site. All three studies were considered key studies by the registrant.

The GLP-compliant Modified Sturm (CO2-evolution) study according to OECD TG 301B showed that the substance is not-ready biodegradable ]. In this ready biodegradability study with non-adapted activated sludge, degradation of the substance amounted to 12 and 34% in the 10 and 20 mg/L treatments after 28 days, respectively. A toxicity control was lacking. Therefore, the results from this study are considered reliable with restrictions and are assigned a Klimisch score of 2.

The GLP-compliant Manometric Respirometry study according to the OECD TG 301F showed that the substance is not ready biodegradable, with degradation after 50 days amounting to a maximum of 2% in spite of using enhanced substance application methods, and the prolonged study duration. Study details are available on the ECHA dissemination website of the substance corresponding to the name Reaction mass of 5-[(2R)-butan-2-yl]-2-[(1R,2R)-2,4-dimethylcyclohex-3-en-1-yl]-5-methyl-1,3-dioxane and 5-[(2R)-butan-2-yl]-2-[(1R,6R)-4,6-dimethylcyclohex-3-en-1-yl]-5-methyl-1,3- dioxane and 5-[(2S)-butan-2-yl]-2-[(1R,2R)-2,4-dimethylcyclohex-3-en-1-yl]-5-methyl- 1,3-dioxane and 5-[(2S)-butan-2-yl]-2-[(1S,2R)-2,4-dimethylcyclohex-3-en-1-yl]-5- methyl-1,3-dioxane and 5-[(2S)-butan-2-yl]-2-[(1S,6R)-4,6-dimethylcyclohex-3-en-1- yl]-5-methyl-1,3-dioxane. Inoculum was freshly activated sludge collected from a predominantly domestic WWTP that was washed three times prior to application, and had a suspended solid concentration of 30 mg/L (dry weight). Test concentration was 100 mg/L. Duplicate test flasks were prepared with direct addition, as well as two enhanced methods, i.e. the use of ultrasound to disperse the test substance and application of the test substance in silicone oil. Oxygen consumption was monitored daily and expressed as percentage of theoretical oxygen demand (ThOD). Since the substance is volatile, evolved carbon dioxide was absorbed to soda lime pellets. Inoculum blank and a toxicity control with the reference substance sodium benzoate were included. The registrant of the substance corresponding to the name Reaction mass of 5-[(2R)-butan- 2-yl]-2-[(1R,2R)-2,4-dimethylcyclohex-3-en-1-yl]-5-methyl-1,3-dioxane and 5-[(2R)- butan-2-yl]-2-[(1R,6R)-4,6-dimethylcyclohex-3-en-1-yl]-5-methyl-1,3-dioxane and 5- [(2S)-butan-2-yl]-2-[(1R,2R)-2,4-dimethylcyclohex-3-en-1-yl]-5-methyl-1,3-dioxane and 5-[(2S)-butan-2-yl]-2-[(1S,2R)-2,4-dimethylcyclohex-3-en-1-yl]-5-methyl-1,3- dioxane and 5-[(2S)-butan-2-yl]-2-[(1S,6R)-4,6-dimethylcyclohex-3-en-1-yl]-5-methyl- 1,3-dioxane noted that an abiotic control was not needed, as the substance is not expected to hydrolyse.. The validity criteria were met. Degradation of the substance amounted 1% after 50 days with direct addition. The use of ultrasound to disperse the substance did not improve biodegradation after 28 days, and increased mineralization only marginally to a total of 2% after 50 days. Application of silicone oils also did not improve biodegradation (mineralization was decreased by 2%) after 28 and 50 days.

There was no toxic effect to the inoculum, as sodium benzoate biodegradation was not altered by the addition of the substance. The results from this study are considered reliable without restrictions and are assigned a Klimisch score of 1. It should be noted that in addition to the two enhanced application methods discussed above, another three enhanced methods were applied using the same test setup, but reported in a separate study report. The results from the latter study are not available on the public dissemination site. This study was considered reliable without restrictions and showed that the three additional enhanced application methods also did not result in degradation of the substance. The fourth study, a GLP-compliant Manometric Respirometry study according to the

OECD TG 302C showed that the substance is not inherently biodegradable, with degradation amounting to 12 and 18% based on O2 consumption, after 28 and 50 days, respectively. The test was conducted with non-adapted activated sludge from a domestic WWTP, and a test concentration of 30 mg/L. The results from this study are considered reliable without restrictions and are assigned a Klimisch score of 1.

**Simulation Tests**

A GLP-compliant aerobic mineralisation study in surface water according to OECD TG 309 is available for the substance. This 60-day study was conducted with natural water and an initial nominal test concentration of 60 μg/L at 22 °C. The registrant reported a halflife of 395 h for the substance at 22 °C, based on (pseudo-) first order kinetics. The registrant did not indicate the reliability of the study.

The evaluating MSCA conducted a thorough reassessment of this study, as several deviations from OECD TG 309 were observed.

According to the original study report, the initial nominal test concentration was 50 μg/L, and not 60 μg/L as reported on the public dissemination site. The OECD TG 309 recommends the use of two test concentrations that differ from each other by a factor of 5 to 10, with the higher and lower test concentration not exceeding 100 and 10 μg/L, respectively. As indicated in the test guideline, there is a risk when testing high concentrations that degradation will not follow first order kinetics and that the first order degradation constant and half-life cannot be estimated. Fortunately, in this study, degradation did seem to follow first order kinetics, and a half-life was derived for the substance.

OECD TG 309 prescribes the inclusion of a blank control, solvent control, sterile control and reference control in the test design. The registrant only included a sterile control. Since the reference control was missing, the viability of the microbial community could not be determined. Considering that the water used in the study was sampled upstream of the registrants activities from an unpolluted river, that the only treatment of the natural water was filtration through a course filter of 100 μm and finally, that the natural water was used on the same day as sampled, the evaluating MSCA supposes that a viable microbial community was present in the water. The registrant also did not assess the toxicity of the substance to the microbial community. Considering that in the above discussed GLP-compliant Manometric Respirometry study, the substance displayed no toxic effects on the inoculum, this is not considered critical. The registrant also did not include a solvent control, but did indicate that the final concentration of the solvent carrier was 0.01% (v/v). Therefore, while the registrant did not include the necessary controls, careful consideration by the evaluating MSCA led to the conclusion that this does not invalidate the study, it merely lowers the reliability. There are two issues that complicated the interpretation of this water simulation study. Firstly, the study was conducted at 22 °C, while the substance is used and released within the context of the REACH Regulation in the EU. The temperature of 12 °C is a default value used in current risk assessment to reflect the average environmental conditions in the EU, and therefore the test should preferably be conducted at 12 °C. Nevertheless, using the Arrhenius equation a DT50 at 12 °C can be extrapolated, and the results can be interpreted. Secondly, the registrant did not use radiolabelled test material. While this is not required by OECD TG 309, it is recommended as a massbalance can be obtained. As no mass balance was available, it remains unclear to what extent the test substance degraded and to what extent it disappeared from the aqueous phase due to evaporation, binding to organic matter in the (turbid) aqueous phase and/or binding to the surface of the test vessels. The study did, however, meet the recovery criterion of 70-110% of nominal at the start of the experiment, and sterile controls were analysed throughout the experiment to quantify disappearance due to processes other than biodegradation. Comparison of the residual concentrations in the test vessels and sterile controls shows that they differed less than 10% for t = 0 h up to and including t = 504 h. This indicates that dissipation up to 504 h was predominantly abiotic. The recently conducted hydrolysis study shows that hydrolysis of the substance in water from the same source (pH 8.2) is slow, as the DT50 at 50 °C amounted to 241 h. Therefore, the evaluating MSCA concludes that the disappearance is most likely due to binding and/or volatilisation of the substance, instead of abiotic degradation.

The registrant estimated a DT50 of 395 h by excluding data points as being in the lag and tailing phase, using only the measurements after 264, 504 and 696 h. This strategy is not supported by the data. Moreover, the tailing phase as referred to in the OECD TG 309, is only applicable to residual activity, in the case of the use of a radiotracer, because of the incorporation of labelled carbon into biomass. The application of this tailing phase to the disappearance of the parent compound is thus not in agreement with the guideline. The evaluating MSCA plotted the natural logarithm of the concentration of the substance in both the sterile controls and in the biotic test vessels against time (Figure 8). The data showed that only when t = 0 is included the plot was not linear for the biotic test vessels, indicating higher than average removal during the first day, i.e. no lag phase. Further, Figure 8 shows that there is no lag phase and no tailing phase. Linear regression with GraphPad Prism (v.6.04) of the data from day 1 until day 60 yielded a *k* of 0.022 (r2 = 0.92) for the biotic test vessels, which corresponds to a DT50 of 31 days at 22°C. The data for the abiotic controls were rather irregular, however, a *k* of 0.010 could be determined (r2 = 0.68), which corresponds to a DT50 of 68 days at 22°C. For both abiotic and biotic test vessels, half-lives are increasing if data from the beginning of the test are omitted from the regression. If the rate constant for the abiotic controls is subtracted from the rate constant for the biotic test, a value of 0.012 remains, which corresponds to a DT50 of 56 days at 22 °C estimated to be only due to biodegradation. Thus, even if all dissipation observed in the test could be attributed to either biotic or abiotic degradation, it is highly likely that at a relevant temperature of 12 °C the DT50 would exceed the vP criterion of 60 days. The median value for an extrapolation of halflives of pesticides in soil to a temperature 10 °C lower is a factor of 2.58 (EFSA, 2007). Applying this default factor yields an estimated DT50 in freshwater due to degradation of 145 days at 12 °C.

The above DT50 of 145 days at 12 °C is a realistic estimation for biodegradation in freshwater. However, this DT50 should be considered indicative, since this study had several limitations, i.e. degradation was monitored only at one test concentration, distinction between degradation and dissipation was hampered by the use of nonlabelled test material, and the experiment was conducted at 22 °C. Therefore, in addition to the realistic DT50 the evaluating MSCA also calculated a best-case DegT50 in freshwater for the substance, including the possibility of abiotic hydrolysis. Firstly, the half-life for hydrolysis at 12 °C was calculated. For the process of hydrolysis, a default factor of 2.2 has been proposed for extrapolation to a 10 °C lower temperature, the corresponding formula being: t½ (X°C) = t½ \* e (0.08 (T – X))) (ECHA, 2014a). Using this default factor and assuming that all of the loss processes in the abiotic controls are accounted for by hydrolysis, the half-life for hydrolysis at 12 °C was estimated to be 152 days compared to 68 days at 22 °C. The degradation in the biotic vessels was subsequently obtained by summing the rate constant for biodegradation at 12 °C (0.0048 d-1) and hydrolysis (0.0046 d-1) yielding an overall rate constant of 0.0093 d-1. This corresponds to a best-case degradation half-life of 74 days at 12 °C. It should be noted that the assumption that all abiotic loss processes are due to hydrolysis is most probably a strong overestimation of the real hydrolysis rate. Another indication for this, comes from the chromatograms presented in the report, that do not show the appearance of the chromatographic peaks of any of the hydrolysis products, probably indicating the complete absence of hydrolysis at ambient temperature and at neutral pH.

Concluding, the evaluating MSCA reassessed the water simulation study, and while deviations from OECD TG 309 were detected, these were not considered sufficiently critical to invalidate the study results. The results are considered valid with restrictions and are assigned a Klimisch score of 2. The realistic DT50 in freshwater for the substance was estimated to be 145 days at 12 °C, while the best-case DT50 in freshwater was estimated to be 74 days at 12 °C.

**Estimated / Predicted data**

|  |  |  |  |
| --- | --- | --- | --- |
| **Type of Evidence / Data Source - Reference** | **Relevance** | **Reliability** | **Adequacy** |
| EPIWIN BioWin v.4.10 | Considered in integration step | Yes, on basis of QSAR assessment of the predictivity of the model taking into account ECHA Guidance provisions for use of this model | Adequate as useful in WoE approach to characterise Biodegradation. |
| CATABOL | Considered in integration step | Considered in integration step | Adequate as useful in WoE approach for estimation of percentage of mineralisation in OECD TG 301C or 301F tests |
| OECD QSAR Toolbox | Considered in integration step | Considered in integration step | Adequate as useful in WoE approach for predicting biotic degradation pathway |
| METEOR | Considered in integration step | Considered in integration step | Adequate as useful in WoE approach for predicting biotic degradation pathway |
| EAWAG-BBD Pathway Prediction System | Considered in integration step | Considered in integration step | Adequate as useful in WoE approach for predicting biotic degradation pathway |

Relevance and reliability are not specifically assessed for the estimated data at this step but the overall quality is further assessed in the next step of weighing the evidence and integration.

For both positional isomers with undefined stereochemistry, biodegradation and

metabolism were estimated using the QSARs listed below. The obtained predictions did

not differ.

**EPIWIN**

Biodegradation can be estimated by the EPIWIN program BioWin v.4.10. The outcome for the Biowin 2 model is 0.0001 and the outcome for the Biowin 6 model 0.0186, which means that the substance is predicted to be not inherently, nor readily degradable respectively. The outcome of the Biowin 3 model for ultimate biodegradation is 2.3809, which corresponds to an estimated mineralization half-life of weeks to months. The exact value would correspond to a half-life in water of 40 days (Aronson et al., 2006). ECHA Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.11, states that a substance is considered as potentially persistent if the value for Biowin 2 or 6 is below 0.5, and the value for Biowin 3 is below 2.25. Borderline cases with Biowin 3 between 2.25 and 2.75 should be carefully examined (ECHA, 2014b).

**CATABOL**

Another program to assess biodegradability is CATABOL. This program estimates the percentage of mineralization in OECD TG 301C or 301F tests. The outcome of the model is 20.8% degradation after 28 days, which means that under the conditions of the OECD 301 tests the substance will only be partly degraded. However, it predicts that primary degradations occur within 28 days, starting with epoxidation of the double bond. It should be noted that 20% mineralization as an experimental result in the OECD 302 series of test guidelines is considered as no mineralization (OECD, 2006). Values between 20-50% ThOD are considered partly mineralized (stable metabolites formed).

**OECD QSAR Toolbox**

The predictions for biotic degradation pathways (microbial) in the OECD QSAR Toolbox (v3.3) show a myriad of possibilities yielding up to 134 possible metabolites. The majority of these metabolites are formed following oxidation of the ring, and/or the alkyl substitution of carbon atoms at different positions. There is no indication of the likelihood of any of these possible transformations given in the Toolbox.

**METEOR**

METEOR is a rule-based expert system used to predict the metabolic fate of a query chemical structure (Marchant et al., 2008). METEOR predicts the oxidation (-OH) of the hexene-ring system and the methyl substituents as most probable (first pass) metabolites for the substance, which is in line with the OECD QSAR Toolbox predictions for microbial metabolism. Dioxane ring opening, through hydrolysis, was not predicted. Plausible metabolites are indicated by a yellow header in the metabolic scheme below, and are for clarity also shown enlarged.

**EAWAG-BBD Pathway Prediction System**

The most detailed prediction of microbial degradation is given by the Pathway Prediction System of the University of Minnesota Biodegradation and Bioremedation Database which now is now hosted at EAWAG, Switzerland (EAWAG-BBD PPS) (Gao et al., 2010). This prediction system shows the most likely (aerobic) degradation pathway to start with oxidation and subsequent ring opening of the 1,3-dioxane ring. The first step, i.e. oxidation in the *ortho* position to the ring oxygen, is thought to be the crucial (rate limiting) step, as the subsequent ring opening steps are considered to be more likely to occur.

The likelihood of the EAWAG-BBD PPS predicted first step is indicated to be “neutral”.

The prediction for the transformation of the 1,3-dioxane ring is predominantly based on the observed microbial transformation of 1,4-dioxane, as documented in Mahendra et al. (2007) (Figure 5). Although the mineralization in the laboratory of 1,4-dioxane is observed in “reinkultures” of monooxygenase expressing bacteria, both in growth supporting as well as co-metabolic mechanisms, this does not necessarily mean that this transformation will also occur in the environment.

On the ECHA dissemination website two ready biodegradability studies are available for 1,4-dioxane (CAS number 123-91-1; EC number 204-661-8). In the Manometric Respirometry test according to OECD TG 301F less than 10% of 1,4-dioxane degraded after 29 days based on oxygen consumption, while in the Headspace Test according to OECD TG 310 less than 5% degraded after 60 days based on CO2 evolution. These studies are reliable, with the first study being assigned a Klimisch score of 2 by the registrants as it was not GLP-compliant, and the latter GLP-compliant test a Klimisch score of 1. From these studies, it can be concluded that 1,4-dioxane is not ready biodegradable. In fact, 1,4-dioxane appears to be poorly biodegradable even after prolonged test duration. In a literature study, the biodegradation potential of 1,4-dioxane in river (n=4), soil (n=13) and activated sludge (n=3) samples was investigated (Sei et al., 2010). Biodegradation of 1,4-dioxane was observed in five out of six soil samples derived from the drainage area of a chemical factory producing 1,4-dioxane (<LOD within 33 days), and in one activated sludge sample via cometabolic degradation in the presence tetrahydrofuran (69% within 14 days). However, the majority of the samples, i.e. 14 out of 20, were not able to degrade 1,4-dioxane at all. Thus, it can be concluded that the potential for 1,4-dioxane degradation is not ubiquitously distributed in natural environment. Based on the above biodegradation studies with 1,4-dioxane, it seems likely that the same holds true for the biodegradation potential of 1,3 dioxane and substituted 1,3- dioxane substances like the substance, as these are thought to biodegrade via the same pathways. Furthermore, the bulky branched alkane substituents at the 5-position (-5- methane-5-butan-2-yl) present in the substance might also form a steric hindrance to the monoxygenase enzyme responsible for the first dioxane ring oxidation step.

The EAWAG-BBD PPS predicted next step is the actual ring opening catalyzed by an isomerase enzyme (not hydrolysis) of the 1,3-dioxane ring, which is indicated to be “very likely”. Following the above described initial oxidation of the 1,3-dioxane ring (step 1), and subsequent ring opening (step 2), the next step (step 3) is another hemiacetal to alcohol and aldehyde transformation by another isomerase enzyme. The remains of the opened 1,3-dioxane ring are split off from the cyclohexene ring in this step.

Based on the above discussion, it is clear that although microbial degradation is possible and observed in lab-studies, the biodegradation studies for 1,4-dioxane show that the oxidation, and subsequent ring opening of cyclic ethers (such as 1,4-dioxane and 1,3- dioxane, and the substance) is not expected to occur in biodegradation simulation studies or to yield significant mineralization rates in the environment.

1. **Integration and Weighing of Evidence & Application of Levels of Confidence**

**4.1 Abiotic Degradation / Hydrolysis**

Overall, the results from the two hydrolysis studies confirm the QSAR predictions that hydrolysis of the substance can occur under acid conditions.

However, this process is very slow. The DT50 at pH 4 and 12 °C is 1830 hours (76 days), but this dissipation may also include other losses such as volatilisation and sorption. The rate of hydrolysis under environmentally relevant pH and temperature could not be determined, most probably because hydrolysis of the substance under these conditions is at best very limited. This was confirmed by the test with river water of pH 8.2 where dissipation in the hydrolysis test at 50 °C was almost a factor two slower than at pH 4. Therefore, the substance is considered to be not hydrolysable, or at most hydrolysis has to be considered negligible under environmental conditions.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Type of Evidence** | **Consistency & Specificity** | **Likelihood/**  **Biological Plausibility** | **Temporality** | **Confidence / Strength of Evidence** | **Remaining Uncertainty** |
| QSAR Toolbox v3.3 Hydrolysis simulator | Consisted prediction with the experimental findings | Plausible | Not relevant | Medium | Low |
| OECD TG 111 Key study | Consistent experimental studies indicating negligible abiotic degradation | Plausible | Not relevant | High | Low |
| OECD TG 111 Supporting study | Consistent experimental studies indicating negligible abiotic degradation | Plausible | Not relevant | Medium | Low |
| **Conclusion from overall confidence** | High/Medium Confidence | | | | |

Confidence level for the QSAR prediction was medium to account for remaining uncertainty for using predicting data as standalone. This does not affect the overall confidence when the prediction is used together with the experimental studies.

Confidence level for the experimental supporting study was medium for reasons outlined in the assessment of the quality of evidence (Klismisch score 3); however, due to consistency with the remaining evidence for this endpoint the confidence level can be regarded as medium.

Overall the available evidence supports one conclusion (no hydrolysis expected under environmental conditions) with high/medium confidence. The interpretation of the available evidence is in line with general principles of chemistry.

**4.2 Biodegradation in water**

Concluding, the screening and inherent biodegradation tests showed that the substance is neither readily nor inherently biodegradable.

Hydrolysis of the substance is at the best very slow, with an estimated DT50 of 76 days at pH 4 and 12.5 °C. Hydrolysis at pH 7 and 9 showed an irregular pattern and no DT50 could be derived for 12 °C. An additional test showed that at 50 °C dissipation in a hydrolysis test at an environmentally relevant pH of 8.2 (river water) was almost a factor 2 slower than at pH 4. It should be noted that the presented hydrolysis study had shortcomings, as the test material was not radio-labelled. Consequently, hydrolysis was most likely overestimated. This was evident from the test conducted at pH 4 and 70 °C where the substance concentration rapidly decreased in the first 24 hours, while the concentration of the proposed hydrolysis product 4 (see Figure 1) only limitedly increased in the same time period. The substance is therefore considered to be only slightly hydrolysable and most likely restricted to acidic conditions only.

Although some biodegradation is predicted by QSAR models, the experimental data show that this partial degradation is very limited. The highest percentage mineralization was observed in the OECD 301B test, but the amount was rather variable (12 to 34% after 28 days). However, it should be realised that this test is in itself not very suitable for a relatively volatile substance such as the substance, although in principle this would result in a lower observed amount of CO2-evolution. In a more recent OECD 301F test including bioavailability enhancements, the amount of mineralization after both 28 and 50 days, is virtually zero, varying between -2 and +2%. In an inherent test according to OECD guideline 302C there was 12% mineralization after 28 days and 18% mineralization after 50 days. These tests show that if mineralization occurs it will still be rather limited, even under the more favourable conditions of the inherent degradability test. The substance is therefore considered to meet the screening criteria for P/vP substances.

Aerobic degradation of the substance was studied in river water with a pH of 8.2. This study has several shortcomings, i.e. some controls were missing, only one concentration was tested, distinction between biodegradation and dissipation was difficult as the test material was not radio-labelled, and the experiment was conducted at 22 °C. Since the substance is used and released within the context of the REACH Regulation in the EU, the test should preferably be conducted at 12 °C, which is considered to reflect the average environmental conditions in the EU. Nevertheless, a thorough reassessment by the evaluating MSCA showed that the results from this study are reliable with restrictions. This study showed that the substance is slowly biodegraded with the DT50 at 22 °C amounting to 56 days. Extrapolated to an EU relevant environmental temperature of 12 °C a realistic DT50 of 145 days was obtained. Even though there were no indications of any significant hydrolysis in this test with river water at slightly alkaline conditions and ambient temperature, a best-case DT50 was also calculated by assuming that all dissipation is caused by either biodegradation or hydrolysis. This yielded a best case DT50 at 12 °C of 74 days.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Type of Evidence** | **Consistency & Specificity** | **Likelihood/**  **Biological Plausibility** | **Temporality** | **Confidence / Strength of Evidence** | **Remaining Uncertainty** |
| Biodegradation Screening test OECD TG 301B | Consistent experimental study indicating the substance is not readily biodegradable | Plausible | Not relevant | Medium | Low |
| Biodegradation Screening test OECD TG 301F | Consistent experimental study indicating the substance is not readily biodegradable | Plausible | Not relevant | High | Low |
| Biodegradation Screening test (OECD TG 301) | Consistent experimental study indicating the substance is not readily biodegradable | Plausible | Not relevant | High | Low |
| Manometric Respirometry Study OECD TG 302C | Consistent experimental study indicating the substance is not inherently biodegradable | Plausible | Not relevant | High | Low |
| Aerobic mineralisation study OECD TG 309 | Consistent experimental study indicating the substance is very persistent in water | Plausible | Not relevant | Medium | Low |
| EPIWIN BioWin v.4.10 | Consisted prediction with the experimental findings. The substance does not biodegrade fast. | Plausible | Not relevant | Medium | Low |
| CATABOL | Predictions for partial mineralisation (stable metabolite formed). | Plausible but experimental data show that partial degradation is very limited. | Not relevant | Low | Low |
| OECD QSAR Toolbox | Predictions for microbial metabolism. | Plausible but experimental data show that partial degradation is very limited. | Not relevant | Low | Low |
| METEOR | Predictions for microbial metabolism. | Plausible but experimental data show that partial degradation is very limited. | Not relevant | Low | Low |
| EAWAG-BBD Pathway Prediction System | Predictions for microbial metabolism. | Plausible but experimental data show that partial degradation is very limited. | Not relevant | Low | Low |
| **Conclusion from overall confidence** | High/Medium Confidence | | | | |

Confidence level for the experimental study (OECD TG 301B) was medium for reasons outlined in the assessment of the quality of evidence (Klismisch score 2); however, due to consistency with the remaining evidence for this endpoint the confidence level can be regarded as medium.

Confidence level for the simulation test (OECD TG 309) was medium for reasons outlined in the assessment of the quality of evidence (Klismisch score 2); however, due to consistency with the remaining evidence for this endpoint the confidence level can be regarded as medium.

1. **Uncertainty Analysis**

No specific uncertainty analysis was considered necessary for this problem formulation. The evidence available were conclusive to decide on the vP properties of the substance when compared to the criteria. The evidence was of good quality, and confidence levels were medium/high.

No additional evidence gathering is considered necessary.

1. **Conclusions**

The screening criterion for persistence (P) is fulfilled for 5-sec-butyl-2-(2,4- dimethylcyclohex-3-en-1-yl)-5-methyl-1,3-dioxane [1], 5-sec-butyl-2-(4,6- dimethylcyclohex-3-en-1-yl)-5-methyl-1,3-dioxane [2] [covering any of the individual stereoisomers of [1] and [2] or any combination thereof]. The results from three biodegradation studies showed that this substance is neither readily, nor inherently biodegradable. Hydrolysis of the substance was shown to be at most very limited at environmentally relevant pH and temperature values. In a river water die-away test, the substance degraded very slowly. This study was conducted with non-radio labelled substance. A best case degradation half-life was estimated by attributing the observed dissipation to either biodegradation or hydrolysis, disregarding processes such as evaporation and binding of this substance. At an environmentally relevant temperature of 12°C this best-case degradation half-life corresponded to 74 days. Therefore, as the vP criterion of 60 days in freshwater is exceeded, the substance is regarded as fulfilling both the P and vP criterion.

## **Example of use of the Weight of Evidence/Uncertainty Template for human health hazard assessment**

**WEIGHT OF EVIDENCE / UNCERTAINTY approach using Mode of Action Framework analysis**

**Mode of Action of DCHP for endocrine mediated irreversible effects to the male reproductive system with the potential to affect male reproductive function and its relevance to Humans.**

**NOTE:**

This example is based on the submission of Annex XV proposal for DCHP from Sweden.

It is only presented here to show how to use the structured Weight of Evidence template.

The original submission within the Annex XV proposal used the WHO/IPCS template for Mode of action analysis / Human relevance, on the basis of which the WoE template has been created.

As indicated in the background document, when Mode of action analysis is performed it is preferred to use the WHO/IPCS template as such since it fits for this type of assessment.

In this example, the relevant sections from the DCHP case have only been copied in the corresponding section of the WoE template without any modification. It is noted that some of the sections of the MoA template are specific to this type of application so there is no direct correspondence to the more generic format of the WoE template.

In addition, the section on assessment of quality of individual lines of evidence is presented in a summarised format in this example since the evaluation was performed in other processes.

**Section 1**

**PROBLEM FORMULATION**

The aim of the analysis is to establish the **Mode of Action of DCHP for endocrine mediated irreversible effects to the male reproductive system with the potential to affect male reproductive function and its relevance to Humans.**

The analysis needs to cover if the effects observed in experimental animals with DCHP are:

* **Species specific**
* **Endocrine mediated**
* **Causing irreversible damage to the male reproductive system and likelihood for adverse effects on fertility**
* **Relevant for humans**

The MoA/HRF (Mode of action / Human Relevance) Framework will be used for the Weight of Evidence analysis following consecutive steps:

1. Hypothesised mode of action statement on the basis of available information
2. Establishment of hypothesised mode of action in experimental species
3. Establishment of human relevance of the established experimental species mode of action

Alternative modes of action will be considered and remaining uncertainty will be recorded.

**Hypothesised Mode of action Statement**

A hypothesis for the potential mode of action of DCHP in male rat reproductive system and the establishment of human relevance includes the following elements:

* DCHP once absorbed is metabolised to the mono-ester MCHP
* MCHP interferes with the Leydig cell testosterone production pathway and/or Sertoli cell function
* Decreased fetal testosterone levels cause male reproductive system effects (Leydig cells, Sertoli cells alterations, epididymis and prostate effects, decreased anogenital distance (AGD)
* The effects seen during *in utero* exposure are also present when the dosing stops (irreversible effects)
* The information available for DCHP follows the same pattern of toxicological effects observed with other phthalates that cause rat male reproduction disorders.
* Considerations of additional modes of action for the low testosterone levels after *in utero* exposure to DCHP e.g. causality involving the hormone-receptor mediated pathways as an alternative/parallell mode of action.
* The rat male reproductive system is similar to humans. Establishment of plausibility of key events occurring in humans based on biological relevance across species.

The following schema presents the hypothesised mode of action:

Hormonal receptors activation

Metabolism to MCHP

Male Impaired Fertility

Placenta Transfer

Male Reproductive system irreversible damage

(Testicular effects, AGD decrease, Hypospadias)

Early Leydig and Sertoli **cells function** alterations *in utero*

Hormonal Changes (Fetal testosterone levels decrease)

**SECTION 2**

**Collection and documentation of all information**

This section summarises the main information used for the establishment of the proposed mode of action for DCHP endocrine mediated male reproductive organ effects (for more information see the following sections).

The data used for the establishment of the hypothesised mode of action of DCHP endocrine mediated reproductive organ effects and male fertility, including human relevance, are available in the Annex I and Section 3. It includes short description of the information, reliability, and the references.

In summary, the available experimental data cover:

* Metabolism of DCHP and species similarities
* Placenta transfer of phthalate metabolites
* Cellular functional alterations of testosterone mediated pathways following *in utero* exposure to DCHP
* Fetal testosterone decrease following *in utero* exposure to DCHP
* Male reproductive tract adverse effects following *in utero* exposure to DCHP (testicular effects, decrease in AGD, hypospadias)
* Male fertility effects (semen quality)
* Estrogen and androgen receptor assays with DCHP
* Similarity of pattern of adverse effects with similar compounds (phthalates)
* Hormonal control of male reproductive system development across species and relevance for DCHP
* Correlation of experimental data for male reproductive organ/system adverse effects with male reproductive system function

The evidence used for establishing the mode of action in experimental species and the human relevance has been collected using the information from the CLH dossier, the registration dossier, as well a search strategy described in Annex I. For the main *in vivo* experimental studies reliability categories have been assigned as described in Annex I. For some evidence we have only used the information available in the abstract from publications (see section 3), this as well as the combination of all lines of evidence has been weighted using the Bradford Hill consideration described in section 3. In this way, levels of confidence using criteria such as consistency, specificity and biological plausibility have been derived. The confidence levels are used as a metric of expert judgment in concluding whether the hypothesised mode of action and human relevance have been established.

**SECTION 3**

**Assessment of quality of individual evidence**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Dicyclohexyl phthalate (DCHP) and its monoester** | | | | |
| **Species** | **Route/Dose** | **Incidence** | **Comments** | **References / Reliability** |
| Rat, Baboon, Ferret, human | Intestinal preparations (*in vitro*) | Species similarity in the metabolism of phthalate diesters between man, rodent, non-rodent and nonhuman primate species. In addition the data indicates that the metabolism to the mono-ester (MCHP) would take place primarily at the intestine. | Abstract only available | Lake BG, Phillips JC, Linnell JC, Gangolli SD. *The in vitro hydrolysis of some phthalate diesters by hepatic and intestinal preparations from various species. Toxicology and Applied Pharmacology, 1977, 39(2): 239-48*  **Used in a WoE approach as supporting study** |
| Rat, Human | Intestinal preparations | The intestinal metabolism of DCHP to MCHP by both rats and human intestine is further confirmed by a study with 16hr incubation of 1%DCHP *in vitro* | Only summary of finding available | IUCLID Datasheet, ECB (as available under ESIS/JRC, currently available within ECHA).  **Used in a WoE approach as supporting study** |
| Rat | Oral | MCHP but not cyclohexanol produced marked testicular atrophy in a rat study | Abstract only available | Lake B.G., Foster J.R., Collins M.A., Stubberfield C.R., Gangoli S.D., Srivastava S.P*. Studies on the effects of orally administered dicyclohexyl phthalate in the rat. 1982, Acta Pharmacol Toxicol (Copenh), 51 (3):217-26*  **Used in a WoE approach as supporting study** |
| Human | *In vitro* system | There is evidence that phthalate monoesters (mMP, mEP, mBP) can cross the placenta and reach the fetus | Abstract only available | Mose T., Mortensen G.K., Hedegaard M., Knudsen L.E. *Phthalate monoesters in perfusate from a dual placenta perfusion system, the placenta tissue and umbilical cord blood*. 2007, Reproductive toxicology, 2007, 23(1): 83-91  **Used in a WoE approach as supporting study** |
| Rat | Oral | Effects on male reproductive system after *in utero* exposure to DCHP, at prepubertal, pubertal and adult stage. Association of hormonal changes with irreversible organ adverse effects | Article published | Aydogan A. M. & Barlas N. *Developmental effects of prenatal di-n-hexyl phthalate and dicyclohexyl phthalate exposure on reproductive tract of male rats: Postnatal outcomes.* Food and Chemical Toxicology, 2013, 51:123-136  **Reliability R1 (se also Annex III)** |
| Rat | Oral | Prenatal male reproductive developmental effects and endocrine association | Article published | Aydogan A. M. & Barlas N*. Influence of in utero di-n-hexyl phthalate and dicyclohexyl phthalate on fetal testicular development in rats.* Toxicology Letters, 2015, 233: 125-137  **Reliability R1 (se also Annex III)** |
| Rat | Oral | Foetal testosterone reduction in GD14 to GD 18 following *in utero* exposure to DCHP (0, 100, 300, 600, 900 mg/kg bw/day) with increasing doses. | Article published | Furr R.J., Lambright S. C., Wilson S. V., Foster M. P. & Gray E. L. *A Short-term in vivo screen using fetal testosterone production, a key event in the phthalate adverse outcome pathway, to predict disruption of sexual differentiation.* Toxicological Sciences 2014, 140(2):403-24 doi:10.1093/toxsci/kfu081  **Reliability R1 (se also Annex III)** |
| Rat | Oral | Decrease in AGD observed in male pups following *in utero* exposure to DCHP in dose response manner. | Article published | Saillenfait, A. M., Gallissot F. & Sabata J. P. *Differential developmental toxicities of di-nhexyl phthalate and dicyclohexyl phthalate administered orally to rats,* Journal of Applied Toxicology, 2009, 29: 510-521.  **Reliability R1 (se also Annex III)** |
| Rat | Oral | Two generation reproductive toxicity study indicating decrease AGD in F1 and F2 | Article published | Hoshino N., Iwai M. & Okazaki Y. *A Two-Generation Reproductive Study of Dicyclohexyl Phthalate in Rats*. The Journal of Toxicological Sciences, Volume 30, Special Issue, 79-96, 2005  **Reliability R1 (se also Annex III)** |
| Rat | Oral | Effects on DNA at prepubertal, pubertal and adult rat testis at the same concentrations as those at which testicular histopathological effects and testosterone levels changes are observed | Article published | Ahbab M.A., Undeger U., Barlas N. & Basaran N. *In utero exposure to dicyclohexyl and di-n-hexyl phthalate possess genotoxic effects on testicular cells of male rats after birth in the comet and TUNEL assays.* Human and Experimental Toxicology, 2014, 33(3): 230-239.  **Used in a WoE approach as supporting study** |
| Rat | Oral | Prolonged preputial separation, reduced AGD, increased areolas/nipple retention, hypospadias, decreased ventral prostate and levator ani/bulbocavernous muscle weight and decreased testicular germ cells were observed in male offspring in the 500 mg/kg bw group | Article published | Yamasaki K., Okuda H., Takeuchi T. & Minobe Y. *Effects of in utero through lactational exposure to dicyclohexyl phthalate p,p’-DDE in Sprague-Dawley rats*. Toxicolcogy Letters 189,2009,14-20.  **Reliability R2 (se also Annex III)** |
| Rat (castrated) | Oral (forced) | 0, 10, 100, 1,000 mg/kg/day  Without (androgenic) and with(antiandrogenic)testosteronepropionate 0.4 mg/kg/day (s.c.).  Negative outcome – neither androgenic nor antiandrogenic activity detected | Results summarised in a report | Hershberger assay – as presented in Table 3-5 page 40 in METI (2002): Current Status of Testing Methods Development for Endocrine Disrupters, Minsitry of Economy, Trade and Industry, Japan.  Available at: <http://www.meti.go.jp/english/report/data/gEndoctexte.pdf>  **Used in a WoE approach as supporting study** |
| In vitro – rat and human testis microsomes | Inhibitory potencies on rat and human 3β- hydroxysteriod dehydrogenase (3β-HSD) and 17β- hydroxysteroid dehydrogenase type 3 (17β- HSD3) activities. | Effect on synthesis of androgens in vitro at μM concentrations.  3β-HSD (IC50s)human = 25.5μM  3β-HSD (IC50s)rat = 24.7μM  17β-HSD3 IC50s human = 8.2μM  17β-HSD3 IC50s rat= 9.1μM  The mode of action of DCHP on 3β-HSD activity was competitive with the substrate pregnelonone but non-competitive with the cofactor NAD+. The mode of action of DCHP on 17β- HSD3 was competitive with the substrate androstenedione but non-competitive with the cofactor NADPH. | Article published | Yuan K., Zhao B., Li, X.-W., Hu G.-X.,Su Y., Chu Y., Akingbemi B.T., Lian Q.-Q. & Ge R.-S. Effects of phthalates on 3β- hydroxysteriod dehydrogenase and 17β- hydroxysteroid dehydrogenase 3 activities in human and rat testes. Chemico-Biological Interactions 195 (2012) 180-188.  Used in a WoE approach as supporting study |
| In vitro – receptor binding to human ERα, ERß, and AR. | Chinese Hamster ovary cell line (CHO-K1) transfected with expression vectors for human ERα, ERß, and AR. | Estrogenic  Relative effective conc. showing 20% of the agonistic activity of 10-9 M 17ß-estradiol) via ERα was 2.8x10-6 M for DCHP.  Antiestrogenic  Relative inhibitory conc. showing 20% of the antagonistic activity of 10-10 M 17ß-estradiol(RIC20) via ERß was 2.5x10-6 M for DCHP.  RIC20 by 10−11M 17ß-estradiol via ERα was 2.8x10-6 M for DCHP.  No androgenic activity.  Antiandrogenic  RIC20 of 10-10 M 5α-dihydrotestosterone via AR was 3.8x10-6 M for DCHP. | Article published | Takeuchi S., Iida M, Kobayashi S., Jin K., MatsudaT. & Kojima H. Differential effects of phthalate esters on transcriptional activities via human estrogen receptors α and β, and androgen receptor. Toxicology 210 (2005) 223–233  Used in a WoE approach as supporting study |
| Rat | Subcutaneous injection – uterotrophic assay in immature females | 2, 20 and 200 mg/kg/bw and day PND 20-22 – no effects on uterine weight. (No information why higher doses were not tested) | Article published | Yamasaki K., Takeyoshi M., Yakabe Y., Sawaki M., Imatanaka N. & Takatsuki M. Comparison of reporter gene assay and immature rat uterotrophic assaya of twenty-three chemicals. Toxicology 170:21-30, 2002.  Used in a WoE approach as supporting study |
| Rat (ovariectomized) | Oral (forced) | 10, 100, 1,000 mg/kg/day without (estrogenic) and with (antiestrogenic) +ethinyl estradiol 30 μg/kg/day | Results summarised in a report | Uterotrophic assay – as presented in Table 3-4 page 36 in METI (2002): Current Status of Testing Methods Development for Endocrine Disrupters, Minsitry of Economy, Trade and Industry, Japan.  Available at: <http://www.meti.go.jp/english/report/data/gEndoctexte.pdf>  Used in a WoE approach as supporting study |

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| **Dibutyl phthalate and other phthalates** | | | | |
| Rat | Oral | Supportive data from other phthalates (DBP) link fetal testosterone insufficiency and abnormal proliferation of Leydig cells in rats |  | Mylchreest E., Madhabananda S., Wallace D. G. & Foster P. *Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(n-butyl)phthalate.* Reprod Toxicol. 2002 Jan-Feb;16(1):19-28.  **Used in a WoE approach as supporting study** |
| Rat and *in vitro* |  | Leydig and Sertoli cells as a target for phthalates | Abstract only available | Jones H.B., Garside D.A., Liu R. & Roberts J.C. *The influence of phthalate esters on Leydig cell structure and function in vitro and in vivo.* Experimental Molecular Pathology, 1993, 58(3):179-93  **Used in a WoE approach as supporting study** |
| Human and rat fetal testes, that were xenografted into castrate male nude mice | Oral | Dibutylphthalate produced no effects in testosterone production in human fetal testes compared to rat fetal testis xenografts | Article publisched | Mitchell R.T., Childs A.J., Anderson R.A., van den Driesche S., Saunders P.T., McKinnell C., Wallace W.H., Kelnar C.J. & Sharpe R.M. *Do phthalates affect steroidogenesis by the human fetal testis? Exposure of human fetal testis xenografts to di-n-butyl phthalate*. Journal of Clinical Endocrinology and Metabolism , 2012, 97(3):E341-8.  Available at: <http://press.endocrine.org/doi/full/10.1210/jc.2011-2411>  **Used in a WoE approach as supporting study** |
| Human, rat and mice fetal testes was xenografted into immunodeficient rodent hosts | Oral (gavage 100, 250 or 500 mg/kg bw for 1, 2 or 3 conscutive days) | Exposure to dibutylphthalate  Only the rat xenograft expressed suppressed steroidogenesis, but all xenograft species exhibited multi nucleated germ cell formations.  Human fetal testes xenograft did not express steroidogenic gene expression. | Article published | Heger N.E., Hall S.J., Sandrof M.A., McDonell E.V., Hensley J.B., McDowell E.N., Martin K.A., Gaido K.W., Johnson K.J. & Boekelheide K. *Human Fetal Testis Xenografts Are Resistant to Phthalate-Induced Endocrine Disruption*. Environmental Health Perspectives, 2012, 120, 1137-1143.  Available at: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3440087/pdf/ehp.1104711.pdf>  **Used in a WoE approach as supporting study** |
| Mouse | Oral via pipette | Postnatal-prepubertal exposure of male mice pups. Di-n-butyl phthalate (DBP) oral doses in corn oil: 0, 1, 10, 50, 100, 250 and 500 mg/kg bw/day from PND 4 – PND7, 14 or 21. Adult mice (exposed PND4-21) were examined for remaining effects at 8wks of age. Study reports inter alia delayed spermatogenesis and impaired Sertoli cell maturation, lower serum and testis testosterone levels, and also reduced AGD. | Article published | Moody S., Goh H., Bielanowicz A., Rippon P., Loveland K.L., & Itman C. *Prepubertal mouse testis growth and maturation and androgen production are acutely sensitive to di-n-butyl phthalate.*  Endocrinology. 2013 Sep;154(9):3460-75. doi: 10.1210/en.2012-2227. Epub 2013 Jun 13.  <http://press.endocrine.org/doi/10.1210/en.2012-2227?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%3dpubmed>  **Used in a WoE approach as supporting study** |
| Rat | Oral gavage | high-dose DBP exposure leads to rapid and reversible diminution of the expression of several proteins required for cholesterol transport and steroidogenesis in the fetal testis, resulting in decreased testosterone synthesis and consequent male reproductive maldevelopment. | Article published | Thompson C.J., Ross S.M. & Gaido K.W., *Di(n-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism.,* Endocrinology, 2004,145(3):1227-37  **Used in a WoE approach as supporting study** |
| Rat | Oral | morphology and incidence of DBP-induced testicular developmental lesion | Article published | Barlow N.J., McIntyre B.S. & Foster P.M., *Male reproductive tract lesions at 6, 12, and 18 months of age following in utero exposure to di(n-butyl) phthalate*. See comment in PubMed Commons belowToxicol Pathol, 2004, 32(1):79-90  **Used in a WoE approach as supporting study** |
| Rat | Oral | Decrease in fetal testosterone levels following exposure to DEHP and DINP | Article published | Borch, J., Ladefoged O., Hass U. & Vinggaard A. *Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal prepubertal and adult male rats*. Reproductive toxicology, 18 (1): 53-61, 2004.  **Used in a WoE approach as supporting study** |
| Rat | Oral | Decrease in fetal testosterone levels following exposure to DEHP and DINP | Article published | Borch J., Metzdorff S., Vinggaard A., Brokken L. & Dalgaard M. *Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis*. Toxicology, 223: 144-55, 2006  **Used in a WoE approach as supporting study** |
| Rat | Oral | dose-response studies with diisobutyl (DIBP), dipentyl (DPeP), dihexyl (DHP), diheptyl (DHeP), diisononyl (DINP), or diisodecyl phthalate (DIDP) indicating that all phthalates, with the exception of DIDP, reduced fetal testicular T production | Article published | Hannas B., Lambright C., Furr J., Evans N., Foster P., Gray E. & Wilson V. *Genomic biomarkers of phthalate induced male reproductive developmental toxicity: a targeted RT-PCR array approach for defining relative potency*. Toxicological Sciences, 125 (2): 544-57, 2012  **Used in a WoE approach as supporting study** |
| Rat | Oral | Effects of dipentyl phthalate on testicular testosterone production | Abstract published | Beverly B., Lambright C., Furr J., Sampson H., Wilson V., McIntyre B., Foster P., Travlos G. & Gray L. *Simvastatin and dipentyl phthalate lower ex vivo testicular testosterone production and exhibit additive effects on testicular testosterone and gene experession via distinct mechanistic pathways in the fetal rat*. Toxicological Sciences, doi: 10.1093/toxsci/kfu149, 2014.  **Used in a WoE approach as supporting study** |

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| **Human relevance** | | | | |
| N/A | N/A | “*a statistically significant change in sperm count in a rodent study is considered to be indicative of a potentiual effect on fertility in humans*” (OECD 2008 due to a greater sperm reserve in rats than in humans. | Guidance document published | OECD. *Guidance document on mammalian reproductive toxicity testing and assessment*. OECD Environment, Health and Safety Publications. Series on Testing and Assessment. No. 43, 2008  **Used in a WoE approach as supporting information** |
| N/A | N/A | *“Decreased AGD in male rats is a hallmark of antiandrogenic substances (Noriega et al 2009; Christiansen et al 2010). A statistically significant change in AGD that cannot be explained by the size of the animal indicates an adverse effect of exposure and should be considered in setting the NOAEL (OECD 2008)”* | Guidance document published | OECD. *Guidance document supporting OECD test guideline 443 on the Extended One-Generation Reproductive Toxicity Test*. OECD Environment, Health and Safety Publications. Series on Testing and Assessment. No. 151, 2013  **Used in a WoE approach as supporting information** |
| Human | N/A | Interlinkage of endocrine mediated pathways with hypospadias | Article published | Silver R.I. *Endocrine abnormalities in boys with hypospadias*.  Adv Exp Med Biol. 2004;545:45-72  **Used in a WoE approach as supporting information** |
| Human | N/A | Interlinkage of hypospadias with fertility | Article published | Kurzrock E.A. & Karpman E. *Hypospadias: pathophysiology and etiologic theories*. Pedriatr Endocrinol Rev. 2004 Mar;1(3):288-95  **Used in a WoE approach as supporting information** |
| N/A | N/A | Review of developmental toxicity of phthalates | Review | Chronic Hazard Advisory Panel on Phthalates and Phthalate Alternatives Final Report , Appendix A, Report to the US Consumer Product Safety Commission, July 2014  Appendix A Developmental toxicity available at: <http://www.cpsc.gov/en/Regulations-Laws--Standards/Statutes/The-Consumer-Product-Safety-Improvement-Act/Phthalates/Chronic-Hazard-Advisory-Panel-CHAP-on-Phthalates/>  **Used in a WoE approach as supporting information** |
| Mammals | N/A | Review of male reproductive development and endocrine pathways | Article published | Nef, S. *Hormones in Male Sexual development*. Genes and Development, 14 (24), 3075-3086, 2000.  **Used in a WoE approach as supporting information** |
| Rat | Oral | Testosterone dependency for male reproductive system development | Article published | Bowman C., Barlow N., Turner K., Wallace D. & Foster P. *Effects of in utero exposure to finasteride on androgen dependent reproductive development in the male rat*, Toxicological Sciences, 74 (2): 393-406, 2003.  **Used in a WoE approach as supporting information** |
| Human | N/A | Interlinkage of hypospadias with fertility | Article published | Asklund C., Jensen T., Main K., Sobotka T., Skakkebaek N., Jorgensen N., *Semen quality, reproductive hormones and fertility of men operated for hypospadias*. International Journal of Andrology, 33: 80-7, 2010  **Used in a WoE approach as supporting information** |
| Human | N/A | Interlinkage of hypospadias with fertility | Article published | Bracka A. *A long term view of hypospadias*. British Journal of Plastic Surgery 42(3): 251-5, 1989.  **Used in a WoE approach as supporting information** |
| Rat | Oral | Testosterone dependency for male reproductive system development | Abstract published | Beverly B., Furr J., Lambright C., McIntyre B., Foster P., Travlos G., Wilson V. & Gray L., *Simvastatin reduces fetal testosterone production and permanently alters reproductive tract development in the male Crl: CD (SD) Rat*, available at: <http://usgov.info/2015/04/16/simvastatin-reduces-fetal-testosterone-production-and-permanently-alters-reproductive-tract-development-in-the-male-rat/>  **Used in a WoE approach as supporting information** |
| Rat | Oral | Antiandrogenic mode of action and male reproductive adverse effects | Abstract published | Wolf C., Lambright P., Mann M., Price M., Cooper R., Ostby J. & Gray L., *Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, P,p-DDE and ketoconazole) and toxic substance (dibutyl- and diethylexyl phthalate, PCB 169 and ethan dimethane suphonate) during sexual differentiation*. Toxicology and Industrial Health, 15: 94-118, 1999.  **Used in a WoE approach as supporting study** |
| Human | N/A | Male reproductive disorders of antenatal origin | Article published | Skakkebaek N.E., Rajpert-De Meyts E. & Main K.M. *Testicular*  *dysgenesis syndrome: an increasingly common developmental disorder*  *with environmental aspects*. Human Reproduction, 2001, 16(5):972-978.  Available at: http://humrep.oxfordjournals.org/content/16/5/972.long |
| Human | N/A | Anogenital distance related effects and fertility | Article published | Eisennberg L.M., Hsieh H.M., Walters C.R., Krasnow R., Lipschultz I. L. *The relationship between Anogenital distance, fatherhood and fertility in adult men*. PLoS ONE 6(5): e18973. Doi:10.1371/journal.pone.0018973  **Used in a WoE approach as supporting study** |
| Human | N/A | Linking of infertility with fetal reproductive systems impairment | Article published | Juul A., Almstrup K., Andersson A., Jensen T., Jorgensen N., Main K., Raipert-De Meyts E., Toppari J. & Skakkebaek N. *Possible fetal determinants of male infertility*. Nature Reviews Endocrinology, 2014, 10: 553-562  **Used in a WoE approach as supporting study** |
| Human | N/A | Anogenital distance related effects and fertility | Article published | Dean A., Sharpe M.R., *Anogenital distance or digit length ration as measures of fetal androgen exposure: relationship to male reproductive development and its disorders*. J Clin Endocrinol Metabl, 2013, 98(6):2230-2238  **Used in a WoE approach as supporting study** |
| Human | N/A | Relevance of testicular dysgenesis syndrome for fertility effects | Article published | Wohlfahrt-Veje C., Main M.K. & Skakkebaek E.N. *Testicular dysgenesis syndrome: foetal origin of adult reproductive problems*. Clinical Endocrinology, 2009, 71:459-465  **Used in a WoE approach as supporting study** |
| Human | N/A | Relevance of testicular dysgenesis syndrome for fertility effects | Article published | Akre O. & Richiardi L., *Does a testicular dysgenesis syndrome exists?* Human Reproduction, 2009, 24(9):2053-2060  **Used in a WoE approach as supporting study** |

**SECTION 4**

**Integration and weighing of evidence (WoE analysis) & Application of levels of confidence**

The hypothesised mode of action as presented in Section 1 above consists of six main key events. All the key events that are listed for the hypothesised mode of action need to be measurable, and are considered essential for the establishment of plausibility and human relevance.

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| ***Key Event 1*** | **Metabolism of DCHP to MCHP** |
| ***Key Event 2*** | **Placenta Transfer** |
| ***Key Event 3*** | **Early Leydig and Sertoli cells function alterations *in utero*** |
| ***Key Event 4*** | **Hormonal changes (**Fetal testosterone levels decrease**)** |
| ***Key Event 5\**** | **Male Reproductive system irreversible damage**  (Testicular effects, Prostate effects, Sperm effects, AGD decrease, Hypospadias) |
| ***Key Event 6\*\**** | **Male impaired Fertility** |

\*For the Key event 5, a number of distinct effects described in the studies available, are grouped together under the general title “male reproductive system irreversible damage”

\*\*The key event 6, includes effects related to semen quality and the finding of hypospadias and AGD decrease in experimental species with expected adverse effects in humans regarding infertility(as part of the testicular dysgenesis syndrome) .

1. **Dose Response Relationships and Temporal Association**

The following table and figure summarise dose response elements from the available data, where it was possible to provide association of the key events with dose response, and temporal association. As the information comes from six different studies, the potency of effects observed indicated with “+” is relative indicating the occurrence of an effect and the severity (not necessarily exactly double or three times higher than the control); the effects reported in this table represent effects that are statistically significant when compared to the control groups.

***Dose response relationship****:* The key events (Key events 3-5) are observed at doses below or similar to those associated with the (adverse) effect. In some case, as the results are from three different studies, not the same parameters have been examined, and therefore information only on one or two key events may be available.

***Temporal association****:* The key events are presented in hypothesised order, starting with effects occurring at fetal stage during *in utero* exposure and continuing manifestation at prepubertal, pubertal and adult stage even without continuation of exposure to the substance after delivery.

The information from the dose response and temporal association is supportive to the fact that the key events (3-5) occur according to the hypothesised order (from earlier to latest key event) in a dose response manner and are irreversible. The information available indicate good quantitative concordance for the experimental species (rat). This information is further used in part c further below.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Key Event 3 | Key Event 4 | Key Event 5 | Key Event 5 | Key Event 5 | Key Event 5 | Key Event 5 | Key Event 5 |
| Study type | Gestation  (treatment) | Gestation  (treatment) | Gestation  (treatment) | Gestation  (treatment) | Prepubertal (recovery) | Pubertal  (recovery) | Adult  (recovery) | F1/F2 Treatment (two generation study) |
| Observation at | Gestation day (GD) 20 | GD 18/ 20 | GD 20 | GD 20 | Postnatal day (PND) 20 | PND 32 | PND 90 | F1: PND 21 and ≥17 wks/F2: PND 21 |
| ***Dose mg/kg bw/day*** | **Early Leydig and Sertoli cells function alterations in**  **utero a** | **Fetal testosterone levels**  **decrease b** | **Male Reproduction system irreversible damage**  Testicular effects  (Leydig cells, Sertoli cells, Epididymis) **c** | **Male Reproduction system irreversible damage d** | **Male Reproduction system irreversible damage**  Testicular/Epididymis effects/Prostate **e** | **Male Reproduction system irreversible damage**  Testicular/Epididymis effects/ Prostate **e** | **Male Reproduction system irreversible damage**  Testicular/Epididymis effects/ Prostate **e** | **Male Reproduction system irreversible damage**  Testicular effects, Sperm effects, Decrease in AGD, increase in areolae  mammae **f** |
| 0 |  |  |  |  |  |  |  |  |
| 10 |  |  |  |  |  |  |  |  |
| 20 | + | ++ | ++ | ++ | ++ | ++ | ++ |  |
| 80 |  |  |  |  |  |  |  |  |
| 100 | ++ | +++ | +++ | ++ | ++ | ++ | ++ | + |
| 250 |  |  |  |  |  |  |  |  |
| 300 |  | +++ |  |  |  |  |  |  |
| 400 |  |  |  |  |  |  |  | + |
| 500 | ++ | +++ | +++ | ++ | ++ | ++ | ++ |  |
| 750 |  |  |  |  |  |  |  |  |
| 900 |  | +++ |  |  |  |  |  |  |

a) Including dose response related reduction in 3β-HSD (Leydig cell function biomarker) and MIS (Sertoli function biomarker) (Aydogan and Barlas 2015)

b) Aydogan and Barlas (2015) for gestation date 20 and Furr et al. (2014) for gestation date 18

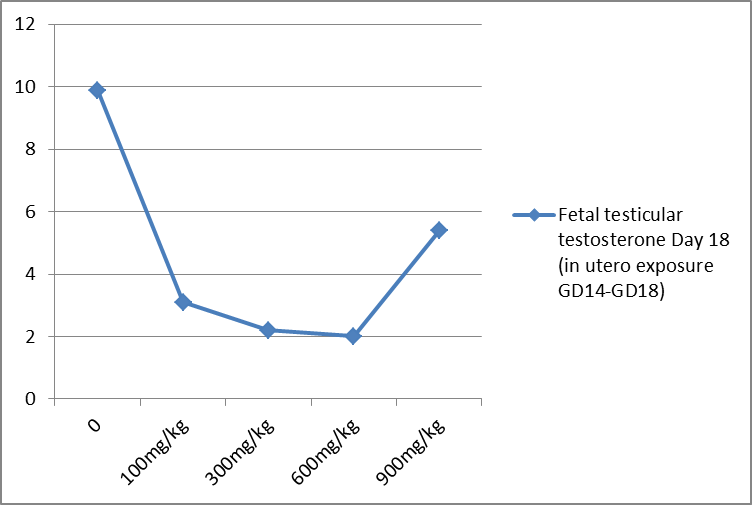
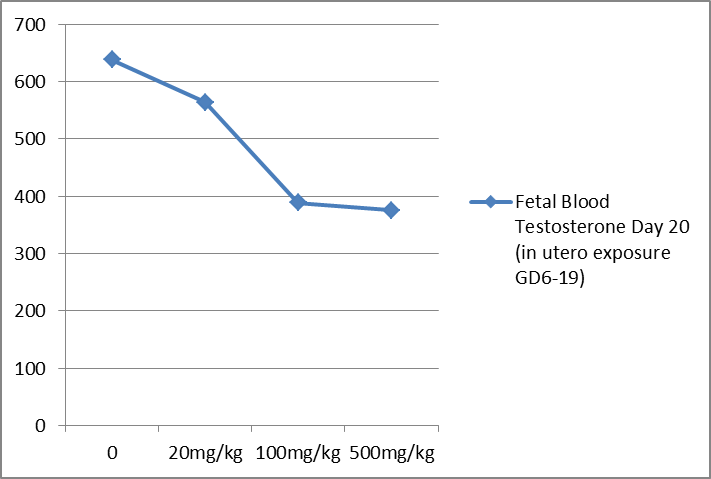
c) Fetal Testicular effects: Atrophic and small seminiferous chords, decreased germ cell numbers in chords, **Sertoli cell** only chords, detached cells from chord wall, multinucleated gonocytes. **Leydig cells**: Increase of medium and large clusters with increasing doses (Aydogan and Barlas 2015).

d) Decrease in AGD (Saillenfait et al. 2009, OECD 2013), Prostate weight reduction (Yamasaki et al. 2009)

e) Aydogan and Barlas (2013): a number of testicular effects are evident in prepuberal and pubertal stages, whereas in the adult stage for some of the observations slight recovery is observed. The treatment occurred only *in utero*. Attached seminiferous tubules and effects on epididymis are however evident also in adult stage, indicating irreversible damage from the *in utero* exposure. In addition prostate relate effects are seen in prepubertal, pubertal and adult stage with atrophic tubules and prostatic intraepithelial neoplasia. This can be seen from plotting results presented in the publication as shown below in figure 1 (the individual observations of parameters for testicular, epididymis and prostate related effects have been pooled to provide an overview of male reproductive organ effects with increasing doses at different time points of revovery).

f) AGD and areola mammae effects occur at lower dose levels in F2 than in F1. (Hoshino et al. 2005, OECD 2013).

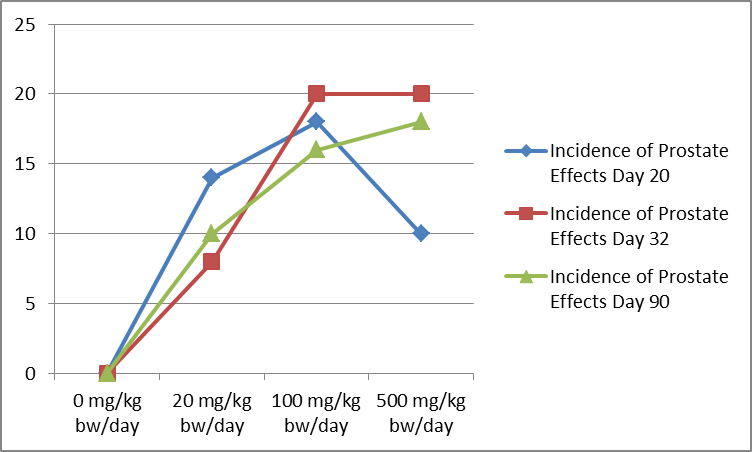
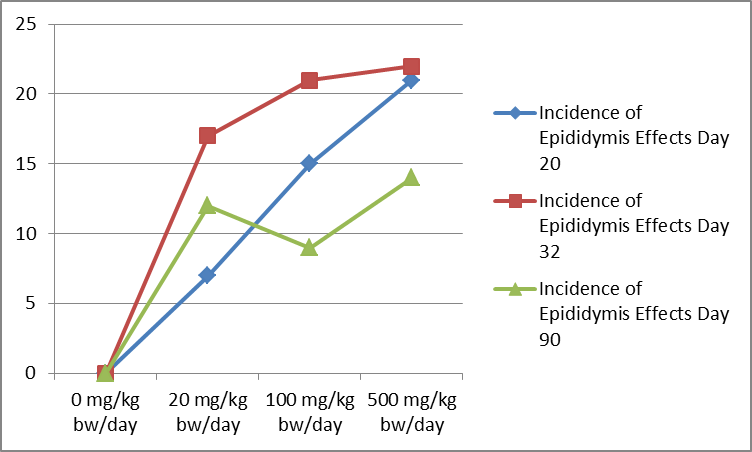
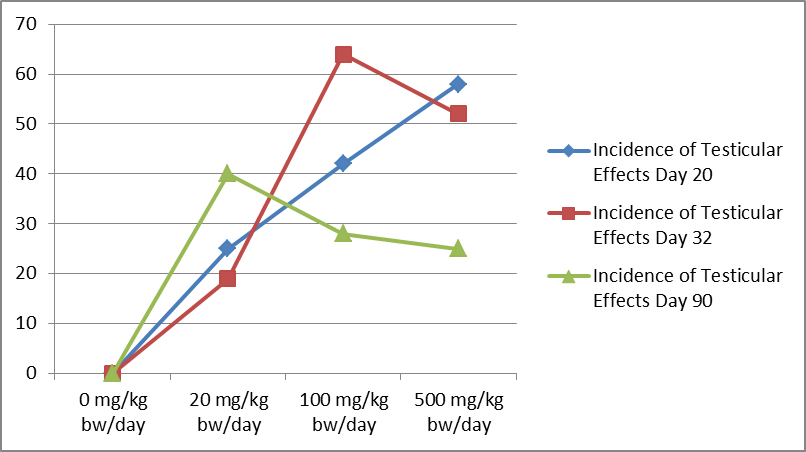
The following figure provides in dose response and temporal association manner the linkage of the decrease of fetal testosterone levels following *in utero* exposure to DCHP with the male reproductive system irreversible damage; individual observations of parameters for testicular, epididymis and prostate related effects have been pooled from the publications available to provide an overview of male reproductive organ effects with increasing doses at different time points of recovery. Fetal testosterone levels from Furr et al. (2014) with treatment at gestational day (GD) 14-18 and measurement of fetal testicular testosterone production *ex vivo* at GD 18 (B) and from Aydogan and Barlas (2015) with treatment gestational day (GD) 6-19 and blood testosterone levels measured at GD 20 (A).

Incidence of testicular, prostate and epididymis effects (pooled data from Aydogan and Barlas 2013) showing irreversibility of effects (*in utero* treatment only) at postnatal day 20, 32 and 90 (C) 

B

A

C



C

C

1. **Consistency & Specificity – Biological Plausibility**

**Consistency & Specificity**

A number of experimental studies with DCHP indicate that the effects in male reproduction system are irreversible following *in utero* exposure. This section addresses that the effects observed for each key event separately are consistent and specific for DCHP as well as that there is causal relationship/linking of the key events with each other and with the toxicological response (key events relationships). It also addresses whether the pattern of effects across species is consistent with the hypothesised mode of action.

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| **Key Event 1 & Key Event 2**  **Metabolism of DCHP to MCHP** |
| **Placenta Transfer** |

The data available, taking into account data and assessments from other phthalates, indicate that metabolism to the monoester is needed for effects to occur. This is supported by available evidence that metabolism to the monoester is likely to occur in humans. According to Lake et al. (1977) a study on the hydrolysis of phthalates, including that of DCHP, showed that there is species similarity in the metabolism of phthalate diesters between man, rodent, non-rodent and nonhuman primate species. In addition the data indicates that the metabolism to the mono-ester (MCHP) would take place primarily in the intestine. The intestinal metabolism of DCHP to MCHP by both rats and human intestine is further confirmed by a study with 16hr incubation of 1%DCHP *in vitro* (IUCLID datasheet, ECB). MCHP but not cyclohexanol produced marked testicular atrophy in a rat study (Lake et al, 1982). There is evidence that phthalate monoesters can cross the human placenta and reach the human fetus (Mose et al: 2007).

*In utero* exposure is most sensitive for the elicitation of the adverse effects in the male reproductive system (Hoshino et al. 2005, Yamasaki et al. 2009, Saillenfait et al 2009, Ahbab and Barlas 2013 and 2015, Furr et al. 2014). Lake et al. (1982) showed testicular damage in adult animals exposed to the higher doses during adulthood. The Hershberger assay has been negative indicating absence of male reproductive effects after short term exposure of adult animals (METI 2002). Thus, a higher sensitivity for damage is present for *in utero* exposure.

Overall, key event 1 and key event 2 are considered essential for the elucidation of the subsequent key events since metabolism is required and placenta transfer to initiate the next key events.

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| **Key Event 3 & Key Event 4**  **Early Leydig and Sertoli cells function alterations *in utero***  **Hormonal changes (**Fetal testosterone levels decrease**)** |
|  |

Testosterone is synthesised within testis from Leydig cells that have a central role in endocrine control of reproduction and the paracrine control of spermatogenesis in mammalian species. The latter is enabled with the contribution of the Sertoli cells in the testis. Functional alterations in Leydig and Sertoli cells (either at cellular or biochemical level) can result in altered testosterone biosynthesis and interference with the normal development of the male reproductive system and its function. Testosterone produced in fetal rat testis by Leydig cells is first detectable at GD15 and reaches a maximum at GD 18-19.

DCHP causes a decrease in fetal testosterone with increasing doses of DCHP, decrease of 3β-HSD enzyme (responsible for testosterone production), as well as decrease in MIS (responsible for regression of Mullerian ducts) and Sertoli cells function biomarker in rats (Aydogan and Barlas 2015). Increasing doses of DCHP also caused impairment of Sertoli cells function and increase of medium and large clusters of Leydig cells in rats (Aydogan and Barlas 2015).

The effect of DCHP on testosterone levels linked to Leydig cells functionality is further supported by *in vitro* data that indicate that DCHP can interfere with testosterone biosynthesis in rats and humans via inhibition of specific enzymes (3β- hydroxysteriod dehydrogenase (3β-HSD) and 17β- hydroxysteroid dehydrogenase type 3 (17β- HSD3)) involved in the biosynthesis of androgens in testes (Yuan et al. 2012).

Foetal testosterone production is dose-dependently reduced in testis tissue examined ex vivo at GD 18 after *in utero* exposure to DCHP (0, 100, 300, 600, 900 mg/kg bw/day) GD14 to GD 18 (Furr et al. 2014). Supportive data from other phthalates (DBP) link fetal testosterone insufficiency and abnormal proliferation of Leydig cells in rats (Mylchreest et al. 2002). In addition it is proposed that in general phthalates alter gene expression for cholesterol transport and steroidogenesis in Leydig cells (Report to the US Consumer Product Safety Commission, July 2014). Furthermore exposure of rats to DEHP and DHP have also been shown to cause decrease in fetal testicular testosterone levels (Borch et al. 2006, Borch et al. 2004, Hannas et al, 2012), providing further supporting evidence of the essentiality of this key event (hormonal changes) in the mode of action analysis that is observed also with DCHP. The effect of another similar substance, DPeP (dipentyl phthalate), on CD mice regarding reduction in fetal testosterone levels (Furr et al. 2014) further supports the absence of significant species differences in relation to key event 4.

Equivocal results on blood hormonal levels (Testosterone, Inhibin B, MIS, FSH, LH) are shown in the Aydogan and Barlas (2013) publication following *in utero* exposure to DCHP, but measuring the hormones at postnatal stages when exposure has ceased. Significant changes are found but not in a dose dependent manner and varying for the different age groups (PND 20, 32 and 90), However, for Inhibin B a reduction in a dose dependent manner in all dose groups and significantly different from the control group in the 100 and 500 mg/kg bw/day adult group (PND 90) are seen. Likewise, the testosterone levels in the 2-generation study (Hoshino et al. 2005) were significantly increased in the F0 middle dose group, but not in the low and high and not in the F1. No firm conclusions can be drawn when these parameters are examined after exposure ceases; as these are most likely earlier key events and their examination within the experimental design should occur at the stage of actual exposure to understand their role in the toxicological adverse pathway.

Furthermore, in a study by Moody et al. (2012) using mice exposed to dibutylphthalate (0, 1, 10, 50, 100, 250 and 500 mg/kg bw/day) prepubertally (PND 4 – PND 7, 14 or 21) lowered testosterone levels were detected in the high dose group, however endocrine effects at lower dose levels were indicated by effects on AGD already at 1 mg/kg bw/day. Reversibility of testosterone levels in adult stage has been shown following *in utero* exposure to dibutyl phthalate, but with male reproductive adverse effects being irreversible in adulthood (Thompson et al. 2004, Barlow et al. 2004). The overall evidence indicates that only fetal testosterone appears to be a sensitive biomarker of the likely initiating events of antiandrogenic properties of DCHP, as changes in serum testosterone outside the critical window of susceptibility (GD 14-18) appear not detectable in dose response manner.

Overall, there is consistency with the available evidence on the occurrence of key event 3 and 4 caused by DCHP *in utero*, as an early key event required to trigger the next key event.

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| **Key Event 5**  **Male Reproductive system irreversible damage** (testicular effects, prostate effects, sperm effects, AGD decrease, hypospadias) |
|  |

Testosterone is essential for the male reproductive tract differentiation in mammalian species (fetal testicular development, epididymis, prostate) (Nef. 2000). Changes of the normal fetal testosterone levels (Key event 4) can result in effects like hypospadias, and decrease in AGD (androgen dependent in male rodents) (Wolf et al. 1999, Bowman et al. 2003)

**Fetal Testicular effects** following *in utero* exposure to DCHP (0, 20, 100, 500 mg/kg bw/day) include:

* Atrophic and small seminiferous chords, decreased germ cells in chords, Sertoli cell only chords, detached cells from chord wall, presence of multinuclear gonocytes (Aydogan and Barlas 2015).

**Decrease in AGD**, has been observed in male pups following *in utero* exposure to 0, 250, 500 and 750 mg/kg bw/day of DCHP in a dose dependent manner (Saillenfait et al. 2009).

A number of **testicular effects** are evident in prepubertal and pubertal stages, whereas in the adult stage for some of the observations recovery is observed. The treatment occurred only *in utero*. Attached seminiferous tubules and effects on epididymis, and increased presence of abnormal sperm are however evident also in adult stage, indicating **irreversible damage** from the *in utero* exposure (Aydogan and Barlas 2013).

The sequence of events does not seem to be reversible when dosing is stopped as indicated by the presence of male reproductive organ effects even at adult stage following only *in utero* exposure (Aydogan and Barlas 2013 and 2015)

The following observations are indicative of irreversible male reproductive systems advese effects:

* Incidence of **testicular effects** (tubular atrophy, germinal cell debris, increase in apoptotic cells) in prepubertal and pubertal stages increase with increasing doses of *in utero* exposure to DCHP. Reversibility in adult stage.
* Incidence of testicular effects (attached seminiferous tubules) increase in prepubertal, pubertal and adult stage with increasing *in utero* doses.
* Incidence of **epididymis effects** (atrophic tubules) increase in prepubertal, pubertal and adult stage with increasing *in utero* doses.
* Incidence of prostate related effects are seen in prepubertal, pubertal and adult stage with atrophic tubules and prostatic intraepithelial neoplasia (Aydogan and Barlas, 2013).

The two generation study showed atrophy of seminiferous tubules and atrophy of testicular changes at 400mg/kg bw/day in F1 generation and **decrease of AGD** in F1 and F2 generation at 400 and at 100, 400mg/kg bw/day, respectively (Hoshino et al. 2005).

The two generation study showed **reduction of spermatids in testis** at 100 and 400mg/kg bw/day in F1 generation, DCHP also affected the **sperm production and maturation** in rats in a dose-dependent manner (Aydogan and Barlas 2013).

In a supportive study, 2 cases of **hypospadias** were found in the high dose group (500 mg/kg bw) (Yamasaki et al. 2009) as well as a decreased AGD, further pointing to effects on the development of the male reproductive organs.

Mitchell et al. (2012) and Heger et al. (2012) reported that DBP exposure of human fetal testes, that were xenografted into castrate male nude mice, produced no effects on testosterone production compared to rat fetal testis xenografts that were exposed to DBP as a positive control. However, this information does not contradict the hypothesised mode of action for DCHP as the xenograft studies do not take into account *in utero* exposure to phthalates throughout the male reproductive organ development (the male programming window) and therefore is not considered strong enough to disregard the proposed mode of action as relevant for humans. In addition, the study from Furr et al. (2014) further confirms that fetal testicular decrease in rats and mice is an early key event with no qualitative species differences observed, but only with some different sensitivity as a result of dose levels of dipentyl phthalate required to trigger the effect.

Overall, there is consistency at the doses at which the later key events appear in relation to earlier key events (see also part a above).

There is consistency in the effects observed (taking also into account similar chemicals e.g. other phthalates) in other species.

|  |
| --- |
| **Key Event 6**  **Male Impaired Fertility** |
|  |

Adverse effects in relation to the development of the male reproductive system and its function are likely to have effects in fertility.

The two generation study with DCHP indicates effects in reproductive organs and function parameters but these effects were not severe enough to render effects on fertility, however, it is known that histopathological changes is a more sensitive indicator of reproductive toxicity than are reduced fertility. Decreased fertility as revealed by effect on fertility index is a rather insensitive endpoint in rats. This may be explained by the rather high sperm reserve available in rats compared to humans (OECD 2008).

Although the experimental evidence of direct impact of DCHP on male fertility is not very strong, there is strong evidence that male reproductive system irreversible effects (e.g. hypospadias, sperm quality effects, decrease in anogenital distance) observed in key event 5 are linked to fertility adverse effects in mammalian species (Asklund et al. 2010, Bracka 1989, Kurzrock and Karpman 2004, Silver 2004, Skakkebaeck et al,.2001). Overall, fetal disturbance of the developing male reproductive system can have multiple effects in mammalian species as described by Skakkebaeck et al. (2001) and summarised as the testicular dysgenesis syndrome (TDS). The disturbed fetal development resulting in alterations in Sertoli cell function and decreased Leydig cell function cause impaired germ cell differentiation and androgen insufficiency; which in turn can lead to reduced semen quality, hypospadias and testicular effects (testicular cancer and testicular descent). The testicular dysgenesis syndrome summarises a number of potential adverse effects due to fetal male reproduction development disturbances but not all of them are always likely to occur concurrently (Akre and Richiardi 2009, Wohlfahrt-Veje et al. 2009).

The available experimental evidence with DCHP point to the direction of potential effects in fertility rather than testicular cancer. This is also further supported by the experimental evidence from similar chemicals (e.g. di-n-butyl phthalate), where testicular cancer in experimental species has not been reported.

The experimental evidence with DCHP on functional alterations of Leydig and Sertoli cells, the fetal testosterone decrease, the testicular histopathological changes and the decrease in AGD, hypospadias and alterations is sperm quality strongly support the hypotheses that impairment of male fertility is likely to occur .

Evidence from human epidemiological studies also show that fetal male reproductive systems development related effects correlate well with low semen quality, disturbances in testosterone levels and histopathological effects, and there are links between infertility observed in men and adverse effects on Sertoli cells and Leydig cells (Juul et al. 2014). Consistency of positive correlation of AGD with sperm count, fertility, testis size and testosterone levels has been reported between human and rats (Dean and Sharpe 2013) further supported by findings that longer AGD in humans can be predictive of normal male reproductive performance in humans (Eisenberg et al. 2011).

Overall, the experimental evidence are consistent with the order the last key event (key event 6) is expected to occur in relation to the previous key events.

There is consistency in the effects observed (taking also into account similar chemicals e.g. other phthalates) in other species.

***Biological Plausibility***

The hypothesised mode of action is supported by general biochemical and pharmacology knowledge on the essentiality of testosterone for the normal development of male reproductive system in all mammalian species (e.g. rodents and humans). Disturbances in the normal levels of fetal testosterone can cause adverse effects in human male sexual development (hypospadias, decrease of AGD, lower semen quality, testicular histopathological changes) which in turn is likely to cause fertility effects (Nef 2000, Dean and Sharpe 2013, Juul et al. 2014, Kurzrock and Karpman 2004, Silver 2004).

The data available does not allow to distinguish whether the target for the metabolite of DCHP is the Leydig cells and/or the Sertoli cells. As the most relevant data are from prenatal toxicity studies, the measurement of the parameters is performed at the final stage of gestation.

It might be that following *in utero* exposure to DCHP, MCHP interferes directly with the biochemical pathway of testosterone biosynthesis in Leydig cells or the initial target is the Sertoli cells (via interference with MIS) which can affect the function of Leydig cells and production of testosterone.

The following table summarises the evidence-based analysis for consistency, specificity and biological plausibility that allow establishing the mode of action in experimental species.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Metabolism of DCHP to MCHP** | **Placenta Transfer** | **Early Leydig and Sertoli cells function alterations *in utero*** | **Hormonal changes (Fetal testosterone levels decrease)** | **Male Reproduction system irreversible damage**  **Testicular effects**  **(Leydig cells, Sertoli cells, Epididymis)** | **Male Impaired Fertility** |
| ***Consistency & Specificity*** | Consistent; Evidence supporting the hypothesised key event | Some evidence provides indication of placenta transfer of formed metabolite. | Consistent; Available data indicating changes at cellular level. | Consistent: Available data shows decrease of fetal testosterone, further supported from knowledge on other phthalate substances | Consistent: Histopathological evidence of testicular effects decrease of AGD, testicular effects observed even at recovery phases, hypospadias in one supportive study | Consistent with general knowledge on impact of male reproduction abnormalities (hypospadias, sperm quality) to fertility; not supported by DCHP treated rat specific information |
| ***Biological Plausibility*** | Plausible | Plausible | Plausible | Plausible | Plausible | Plausible |

1. **Qualitative and Quantitative Human Concordance**

In this section, the established mode of action is presented in a Weight of Evidence process indicating the confidence levels, as derived by the use of the Bradford Hill considerations (see previous sections). In addition it presents the likelihood (plausibility) of human relevance in a qualitative manner and quantitative manner (for some of the key events in experimental species) (in the absence of DCHP specific human data).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | ***Qualitative Concordance*** | |  | ***Quantitative Concordance*** |  |
| ***Key Event (name)*** | ***(Evidence in Experimental Species)*** | ***(Evidence in Humans)*** | ***Confidence*** | ***(Evidence in Experimental Species)*** | ***Confidence*** |
| **Metabolism of DCHP to MCHP** | Evidence based | Plausible and some evidence based | High |  |  |
| **Placenta Transfer** | Likely, sufficient evidence available | Plausible | Medium\* |  |  |
| **Early Leydig and Sertoli cells function alterations *in utero*** | Evidence based | Plausible and some evidence based | Medium \*\* | Evidence based (see section 5a) | Medium |
| **Hormonal changes**  **(Fetal testosterone levels decrease)** | Evidence based | Plausible | High | Evidence based (see section 5a) | High |
| **Male Reproduction system irreversible damage**  **Testicular effects**  **(Leydig cells, Sertoli cells, Epididymis)** | Evidence based | Plausible | High | Evidence based (see section 5a) | High |
| **Male Impaired Fertility** | Likely, not chemical specific (DCHP) based | Plausible | Medium\*\*\* |  |  |

Overall, the weight of evidence is sufficient to establish the hypothesised mode of action in experimental species.

There are no fundamental qualitative differences in key events observed in experimental species and those expected in humans. Human relevance cannot be excluded due to kinetic and dynamic factors.

\*Some remaining uncertainty as there is not extensive experimental evidence on quantitative aspects of placental transfer; however the presence of the treatment related adverse effects are clear. *In vitro* evidence suggestive of interference of DCHP with steroidogenic enzyme activity in testis both in rats and in humans (Yuan et al. 2012).

\*\* Some remaining uncertainty on whether the adverse effect is due to interference with testosterone biosynthesis or with functional changes of Leydig or Sertoli cells. It may also be through a combination of effects on these cell types.

\*\*\* Some remaining uncertainty regarding the evidence available for impairment of fertility in experimental species and human relevance. Although there are no effects observed in fertility parameters in a two generation reproductive study the observed effects on sperm count are indicative of an adverse effect in humans (OECD 2008). Considering the reproductive capacity of rats, decreased fertility as revealed by effect on fertility index is a rather insensitive endpoint. This may be explained by the rather high sperm reserve available in rats compared to humans (OECD 2008). Difference in outcomes for the sperm parameters between the 2-generation study (Hoshino et al. 2005) and the Aydogan and Barlas studies (2013 and 2015) may depend on the different periods of exposure and use of different species of rats. It could be hypothesised that the longer exposure time in the 2-generation study caused defective germ cells to completely disappear, while the more short term exposure (GD 6-19) left damaged cells still viable to produce sperm of lower quality. The available evidence for qualitative correlation of male impaired fertility with the previous key events indicate plausibility on the basis of strong evidence (not DCHP specific) as described for Key event 6 in part b above.

1. **Other potential Modes of Action**

**The following modes of action have been considered as part of the weight of evidence analysis within the context of establishing specificity and biological plausibility of the observed adverse effects. It is concluded that the following modes of action are not likely to occur based on the available data and the existing knowledge on these pathways.**

* *Receptor Mediated Pathways*

Endocrine mediated effects involved in the male reproductive system development could include receptor mediated pathways. However in the case of DCHP, the available *in vivo* data (Hersberger (METI 2002) and Uterotrophic assays (Yamasaki et al. 2002), METI 2002) indicate that this mode of action is not plausible in the absence of any significant estrogen or androgen effects. This is further supported from data on other phthalates that also show no *in vivo* receptor (androgen or estrogen) activity.

DCHP shows *in vitro* estrogenic, antiestrogenic and antiandrogenic effects through binding to ERα, ERβ and AR (Takeuchi et al. 2005). However, since negative results from one Uterotrophic assay (Yamasaki et al. 2002) and one Hershberger assay (METI 2002) have been reported, effects mediated directly through receptor binding are less likely to be the main source of the adverse effects observed.

* *Cholesterol Biosynthesis/Availability Pathway*

Competition of cholesterol related pathways during fetal development is more likely to occur at a stage relevant only of the normal development of the fetal male reproductive system. Cholesterol is essential during pregnancy for the development of the fetus as it is the precursor of steroidogenesis. However since no effects are observed in females in the 2-generation study (Hoshino et al. 2005), it is less likely that DCHP interferes either with the availability/transfer of cholesterol via the placenta, or with the first biochemical reactions of conversion to pregnenolone to a greater extent. However this would not exclude potential interference with cholesterol/testosterone biochemical pathway triggered through interference with fetal male specific receptors or pathways; for the latter there is no conclusive evidence for DCHP to support the elaboration of key events that would occur earlier to the functional changes at cellular level or testosterone biosynthesis (key events 3 and 4 of the hypothesised mode of action). Cholesterol lowering drugs have been shown to lower fetal testosterone levels in rat with additive effect when co administered with dipentyl phthalate (Beverly et al. 2014) as well as linking with reproductive tract development alteration in rats (Beverly et al. 2015).

* *Pituitary / Hormonal regulation*

Involvement of pathways that include pituitary regulation are less likely. DCHP does not cause any effects without *in utero* exposure; in the Hershberger assay no effects were seen suggesting absence of effects involving regulation of testosterone from the pituitary axis.

* *Genotoxicity Mediated Pathways*

Consideration of genotoxicity on testicular cells of male rats following *in utero* exposure to DCHP has also been investigated (Ahbab et al. 2013). However it is unlikely that a genotoxic mode of action for DCHP is involved as the observations on DNA damage are observed at the same time points as the ones involved in the testosterone biosynthesis mode of action proposed. It is likely that the genotoxicity is secondary to the cellular effects observed. There is no other data from similar substances to support genotoxic potential as an alternative mode of action. In addition, the overall data set including the two generation reproductive toxicity assay do not reveal any results that could be associated with an irreversible genotoxic mode of action.

**SECTION 5**

**Uncertainty Analysis**

There are no major data gaps identified in establishing the mode of action. The data and the analysis indicate that DCHP *in utero* exposure interferes with steroidogenesis of the male reproductive system development, and an endocrine mediated mode of action is responsible for the adverse effects observed that are of irreversible nature. The mode of action is based on evidence in experimental species and is found plausible with high confidence for humans.

Remaining uncertainty regarding the exact temporal relation of the cellular key events involved could be resolved with further research *in vitro* to identify if MCHP competes with the regular biochemical pathway of testosterone biosynthesis (cholesterol male mediated pathways) or the effects are due to functional changes of Sertoli cells that in turn affect the testosterone production from Leydig cells or other modes of action.

**SECTION 6**

**Conclusions**

The hypothesised mode of action of DCHP for endocrine mediated irreversible effects to the male reproductive system with the potential to affect male reproductive function and its relevance to humans has been established with medium/high confidence. The overall weight of evidence analysis shows that the male reproductive effects observed following *in utero* exposure to DCHP are mediated via an endocrine (antiandrogenic) mode of action that involves irreversible effects induced by alterations in steroidogenesis in fetal life. This is supported by the available experimental evidence, the biological plausibility for human relevance and the absence of inconsistent evidence.

**Annex I – Method for collection and assessment of the data used for preparing SVHC-dossier for dicyclohexyl phthalate (DCHP)**

The information used in the CLH-report and the RAC-documentation for the classification of DCHP was used as the starting material. An additional search was done in PubMed July 2014, complemented in June 2015, using the search term: dicyclohexyl phthalate. Additional information on similar substances (other phthalates) as available in registration dossiers, in previous RAC opinions as well as existing regulatory reviews of these substances were considered as part of the strategy of collecting the available information.

In this Annex, the reliability of the *in vivo* studies considered for the preparation of the SVHC dossier is further elaborated. The reliability of other types of information (*in vitro* studies, bridging information from other phthalates) is addressed with confidence levels using the Weight of Evidence approach within the Mode of Action/ Human Relevance Framework on the basis of the Bradford Hill considerations, for more information see Annex II

The studies were all considered relevant as already used by RAC and/or fulfilling the relevance criteria of the Science in Risk Assessment and Policy (Scirap) tool. The used *in vivo* studies were evaluated using Scirap tool available at [www.scirap.org](http://www.scirap.org). The tier I and II tool was used to evaluate the reliability of the studies.

During the preparation of the dossier contact was taken with authors for complementary information if the papers contained crucial information with some flaws in the reporting with regards to the study design. This made it possible to better evaluate the reliability of the study results.

The reliability categories used are adapted from the Klimisch score by Moermond et al. (submitted manuscript) and defined as follows:

**R1 Reliable without restrictions**: All critical reliability criteria for this study are fulfilled. The study is well designed and performed, and it does not contain flaws that affect the reliability of the study.

**R2 Reliable with restrictions**: The study is generally well designed and performed, but some minor flaws in the documentation or setup may be present.

**R3 Not reliable**: Not all critical reliability criteria for this study are fulfilled. The study has clear flaws in study design and/or how it was performed.

**R4 Not assignable**: Information needed to make an assessment of the study is missing. This concerns studies which do not give sufficient experimental details and which are only listed in abstracts or secondary literature (books, reviews, etc.), or studies of which the documentation is not sufficient for assessment of reliability for one or more vital parameters.

**Table**. Summary of the evaluations on *in vivo* DCHP publications

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reference** | **Study design** | **Scirap summary**  **(tier II)** | **Comments** | **Final category** |
| **Hoshino N., Iwai M., Okazaki Y. *A Two-Generation Reproductive Study of Dicyclohexyl Phthalate in Rats*.**  **The Journal of Toxicological Sciences, Volume 30, Special Issue, 79-96, 2005** | OECD TG 416 (1983) + additional ED endpoints | Of the 29 applicable parts of the evaluation, 62% fulfilled, 14 % partially and 24%not fulfilled – overall good reporting, the not fulfilled regards e.g. analysis of feed and water for contaminants. | Information about study design was also available in the introductory paper of the special issue: Yamasaki K., Takahashi M, Yasuda M. 2005. Two-Generation Reproductive Toxicity Studies in rats with extra parameters for detecting endocrine disrupting activity: Introductory overview of results from nine chemicals. The Journal of Toxicological Sciences, Volume 30, Special Issue, 1-4, 2005 | Over-all R1 |
| **Yamasaki K., Okuda H., Takeuchi T., Minobe Y. *Effects of in utero through lactational exposure to dicyclohexyl phthalate p,p’-DDE in Sprague-Dawley rats*. Toxicolcogy Letters 189,2009,14-20.** | Mated female rats (F0) (~12 weeks old) subdivided into 4 equally sized groups  • Culling at PND 4, to litter size of 8 aiming for 4 pups/sex when possible.  •At weaning pups (F1) randomly subdivided into sub groups A and B  A. Sacrificed at 10 weeks of age.  B. 2 females and 2 males/dam mated at 12 weeks to assess reproductive performance and possible effects on early embryonic development (caesarean sections performed on gestation day 13). | Of the 28 applicable parts of the evaluation, 43% fulfilled, 18 % partially and 39% not fulfilled – the not fulfilled depending lack of detailed reporting regarding animals, housing, feed, and choice of administration route. | Important study results – no contact taken with author as other studies with similar design show similar results. Results used as supportive | R2 |
| **Saillenfait, A. M., Gallissot F., Sabata J. P., *Differential developmental toxicities of di-nhexyl phthalate and dicyclohexyl phthalate administered orally to rats*, Journal of Applied Toxicology, 2009, 29: 510-521.** | Study protocol resemble Prenatal developmental toxicity study (OECD TG 414). 20-25 pregnant dams/group.  In addition Anogenital distance was measured on GD 21.  Satellite study  6-9 animals/dose level, dosing interval as main study, for examination of liver effects (histopathology, enzyme activity and liver weights) on GD 21.  study. | Of the 30 applicable parts of the evaluation, 74% fulfilled, 13 % partially and 13% not fulfilled – overall good reporting, the not fulfilled regard analysis of feed and water for contaminants. |  | R1 |
| **Aydogan A. M., Barlas N. *Developmental effects of prenatal di-n-hexyl phthalate and dicyclohexyl phthalate exposure on reproductive tract of male rats: Postnatal outcomes*, Food and Chemical Toxicology, 2013, 51:123-136** | Pregnant rats  After delivery all pups were allowed to grow with their dam for 1 month and then male pups were separated and housed 4/cage until they were killed on:  - PND 20 (pre-pubertal),  - PND 32 (pubertal)  - PND 90 (adult). | Initial evaluation - Of the 29 applicable parts of the evaluation, 57% fulfilled, 7 % partially and 34.5% not fulfilled – reporting poor for animals, housing and feed. | Further info provided from author – renewed evaluation: Of the 28 applicable parts of the evaluation, 82% fulfilled, and 18% not fulfilled. | Over-all R1 |
| **Aydogan A. M., Barlas N. *Influence of in utero di-n-hexyl phthalate and dicyclohexyl phthalate on fetal testicular development in rats*. Toxicology Letters, 2015, 233: 125-137** | Wistar rats, exposed GD 6-19 with DCHP 0, 20, 100 and 500 mg/kg bw/day. Male foetuses examined on GD 20 for testosterone, FSH, inhibin B, MIS, testis histopathology (including immunohistochemical staining for 3β-HSD. MIS/AMH. AR and PCNA and determination of Leydig cell numbers and clusters. | Of the 29 applicable parts of the evaluation, 69% fulfilled, and 31% not fulfilled - reporting poor for animals, housing and feed. | Further info provided from author – renewed evaluation: Of the 28 applicable parts of the evaluation, 82% fulfilled, and 18% not fulfilled. | Over-all R1 |
| **Furr R.J., Lambright S. C., Wilson S. V., Foster M. P., Gray E. L. A *Short-term in vivo screen using fetal testosterone production, a key event in the phthalate adverse outcome pathway, to predict disruption of sexual differentiation*. Toxicological Sciences 2014, 140(2):403-24 doi:10.1093/toxsci/kfu081** | *In vivo* screen to detect disruption of fetal testosterone synthesis | Of the 29 applicable parts of the evaluation, 83% fulfilled, 6.9 % partially and 10% not fulfilled |  | R1 |