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Revised Guidance Document on Developing and Assessing Adverse Outcome Pathways

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No. 184

Second Edition of the Guidance Document, replacing the original version dated 2013.

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Series on Testing and Assessment

No. 184

Revised Guidance Document on Developing and Assessing Adverse Outcome Pathways

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2017

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Series on Testing and Assessment
No. 184

GUIDANCE DOCUMENT ON DEVELOPING AND ASSESSING ADVERSE OUTCOME PATHWAYS

- Second Edition -

FOREWORD

The Adverse Outcome Pathway (AOP) methodology is an approach that provides a framework to collect, organise and evaluate relevant information on biological and toxicological effects of chemicals. More specifically, the AOP approach organises existing knowledge concerning biologically plausible and empirically supported links between molecular-level perturbation of a biological system and an adverse outcome at a level of biological organisation of regulatory concern. This approach supports the use of a mode (and/or mechanism) of action basis for understanding the adverse effects of chemicals and other stressors. This guidance document provides an introduction to the vocabulary, concepts, and insight into the development of an AOP, including identification and use of relevant scientific data and resulting knowledge. The document also briefly outlines some potential regulatory uses of AOPs. At the end of the document there is a glossary that aims to facilitate understanding of the AOP concept and its components, as well as its ultimate application.

A complementary document, the OECD AOP Users' Handbook, provides in-depth information on AOP development and is a more appropriate document for those looking for specifics on how to build their AOPs (OECD, 2016a). The AOP Users' Handbook also includes some guidance for evaluation of confidence in the underlying information. The OECD guidance document on the use of AOPs in developing integrated approaches to testing and assessment (IATA) (OECD, 2016b) explains how the AOP concept can be applied as a framework to develop IATA for different purposes. As the number of documented AOPs increases further demonstrations can be made, and guidance developed, of their application in IATA and also their use in various regulatory contexts. As experience grows, it is expected that guidance documents for the development of IATAs based on AOPs as well as harmonised IATAs will be developed.

This guidance document was prepared initially in December 2012 by the Secretariat in collaboration with the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). At that time it was acknowledged that the guidance should be revised once expert groups and member countries acquire experience in developing and assessing AOPs. Since then, some experience has been gained in developing AOPs, and a Users' Handbook supplement to the guidance document for developing and assessing AOPs was published. The Users' Handbook replaces the AOP Template (Part II of the original guidance) and the sections dealing with Data Summation, AOP assessment and Confidence in an AOP from the original guidance. The Users' Handbook was developed to include material related to the AOP-Wiki and the weight of evidence (WoE) considerations that will continue to evolve and will require regular updating. In contrast, the present guidance document deals with those areas of the AOP framework that are unlikely to change while moving forward. This second edition of the Guidance Document provides an historical background for the AOP development programme, and outlines the elements required to construct an AOP as well as the principles of the AOP framework.

The Second Edition of the Guidance Document was approved by the 29th Meeting of the WNT in April 2017. This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

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BACKGROUND

1. The historical paradigm for protecting humans and the environment from adverse effects of chemicals has centred primarily on whole animal toxicity testing with single chemicals of concern. However, due to the costs and time involved, it is not practical or feasible to test exhaustively all chemicals that could adversely affect humans and ecosystems. These realities indicate the need for scientifically defensible models and tools for more efficiently predicting adverse effects of chemicals. To date, our limited knowledge about biological systems has hindered efforts to use mechanistic information as a basis for effects extrapolation. Despite this, advances in toxicogenomics, bioinformatics, systems biology and computational toxicology offer potential in this context (NRC, 2007; Krewski et al., 2010). The Adverse Outcome Pathway (AOP) has been proposed as a framework to collect and evaluate relevant chemical, biological and toxicological information to support advancing more efficient and predictive assessment and testing strategies. The framework organises information as a basis to support the use of mode (and/or mechanism) of action for better understanding the path to adverse effects. It links existing and new methods with systems biology. Briefly, consideration of weight of evidence (WoE) for AOPs builds on concepts and principles incorporated in pre-existing evolving frameworks for mode of action (MOA)/human relevance analysis involving large numbers of scientists internationally, as was reported in Meek et al. 2014a.

2. The primary purpose of this guidance document is to provide an introduction to the development and assessment of AOPs, and the framework for consistent information gathering and organisation, including definitions for AOP-specific terminology. In this context, an AOP is a conceptual construct that portrays existing knowledge concerning the pathway of causal linkages between a molecular initiating event (MIE) and a final adverse outcome (AO) at a biological level of organisation that is relevant to a regulatory decision (Ankley et al., 2010).

INTRODUCTION

3. Recognising the limitations of current testing approaches for toxicological assessment and the rapid development of new biochemical and cellular assay systems and computational predictive methods, regulators and other stakeholders have been exploring ways to integrate existing knowledge from *in vivo* tests with the results of alternative methods and other sources of information, as a basis to evolve testing and assessment strategies to increase efficiency and predictivity.

4. Over the past two decades, a variety of groups have advocated systems- and pathway-based approaches to define the processes by which toxicants elicit AOs of interest for public health. Early applications of the pathway approach were often referred to as exposure-dose-response models or biologically based dose-response models (Clewell et al., 1995; Shuey et al., 1995). In 2001, a framework for using MOA information to determine human relevance of animal data was published by the International Programme on Chemical Safety (IPCS) (Sonich-Mullin et al., 2001) based on division of the path from metabolism to effect into a series of key events. The latter was adopted by the OECD in 2002 (OECD, 2002). In 2007, the United States National Academy of Science (NAS) published the *Report on Toxicity Testing in the 21st Century: A Vision and a Strategy* in which the concept of a 'toxicity pathway' based on similar principles was proposed (NRC, 2007). The report included a vision to reorient toxicity testing to evaluate perturbations of biological pathways by chemical exposures in well-designed *in vitro* methods using cells, preferably of human origin.

5. Since the McKim Conferences on Predictive Toxicology in 2006, 2007, and 2008 (http://mckim.qsari.org), and in parallel with refinement of the IPCS MOA framework (Boobis et al., 2006; 2008), the term "Adverse Outcome Pathway" (AOP) evolved (http://mckim.qsari.org). It was originally introduced by Ankley and co-workers (Ankley et al., 2010) and has evolved in the context of the OECD program as a conceptual construct that portrays existing knowledge concerning the pathway of causal linkages between a MIE and a final AO at a biological level of organisation that is relevant to a regulatory decision. In an AOP, it is important to integrate the known information from various sources. The approach is based on the concept that toxicity results from the chemical first reaching and then interacting with an initial target or targets in the organism. As such, an AOP is the sequential progression of events from the MIE to the *in vivo* outcome of interest (Figure 1). Generally, it refers to a broader set of pathways that would: 1) proceed from the MIE, which represents the perturbation that results from the interaction of a stressor with a biological target (e.g. DNA binding, protein oxidation, etc.); 2) continue on through a sequential series of biological activities (e.g. gene activation, or altered tissue development, etc.) that are essential for progression of the toxicity; and 3) ultimately culminate in the final AO relevant to human or ecological risk assessors (e.g., mortality, disrupted reproduction, cancer, or extinction, etc.) (OECD 2011, ENV/JM/MONO(2011)8).

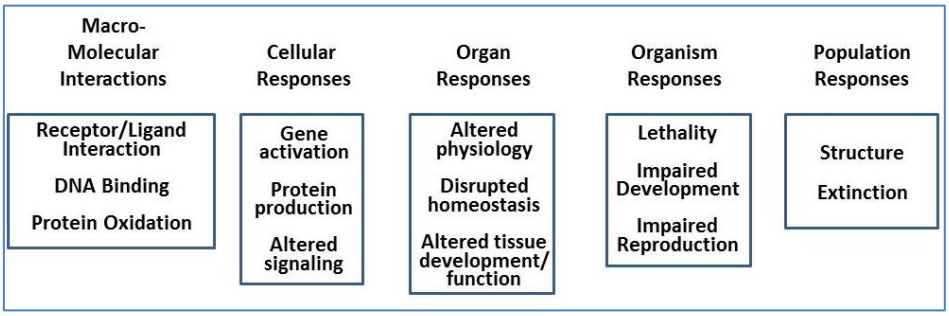

Figure 1. A schematic representation of the Adverse Outcome Pathway (AOP) is illustrated with reference to a number of examples at different levels of biological organisation.

6. The concept of AOPs has evolved based on a number of discussions in recent years, including a Pellston Conference convened by the Society of Environmental Toxicology and Chemistry in 2009 (Villeneuve and Garcia-Reyero, 2011; Watanabe et al., 2011; Perkins et al., 2011; Nichols et al., 2011; Celander et al., 2011; Kramer et al., 2011; ENV/JM/MONO(2012)10/PART1; ENV/JM/MONO(2012)10/PART2; Enoch and Cronin 2010; ENV/JM/MONO(2011)6; Schultz et al., 2011; Hill, 1965; US EPA,2005; US EPA, 2011). Papers produced from these discussions deal with a variety of aspects of AOPs including their derivation from existing data to techniques for reverse-engineering AOPs from genomics data. A Workshop organised by the OECD entitled Using Mechanistic Information in Forming Chemical Categories was held in December 2010 in Washington DC and resulted in a number of recommendations and conclusions for the near term (i.e., subsequent two years). These recommendations were to:

1) engage toxicologists and other scientists in discussions of AOPs in an effort to foster interactions by developing AOPs for well-established effects (e.g., skin sensitisation);
2) complete the proof of concept that began with the December 2010 workshop by developing AOPs for several different longer-term human health and ecotoxicological endpoints;
3) develop a strategic plan for identifying, assessing and advancing AOPs and their integration into the OECD QSAR Toolbox, including development of:
    a) an information template that can be used for developing and assessing AOPs,
    b) a set of guiding principles for assessing the completeness and acceptance of an AOP, and
    c) a format for attaining mutual acceptance of an AOP;
4) harmonise the terminology associated with AOPs (OECD 2011, ENV/JM/MONO(2011)8).

7. In response to recommendation (1) and (2), the OECD developed an AOP for protein binding leading to skin sensitisation. Figure 2 presents the flow diagram of the pathways associated with skin sensitisation initiated by covalent binding to proteins (OECD 2012, ENV/JM/MONO(2012)10/PART1; ENV/JM/MONO(2012)10/PART2). Based on experience acquired in development of this AOP and in an effort to address recommendations (3) and (4), an initial version of the current Guidance was developed. This earlier Guidance has been refined here to address the essential elements and principles for developing and assessing AOPs. More details are included in the Users' Handbook, which is designed to be updated more easily than this guidance, based on feedback received from AOP developers and assessors.

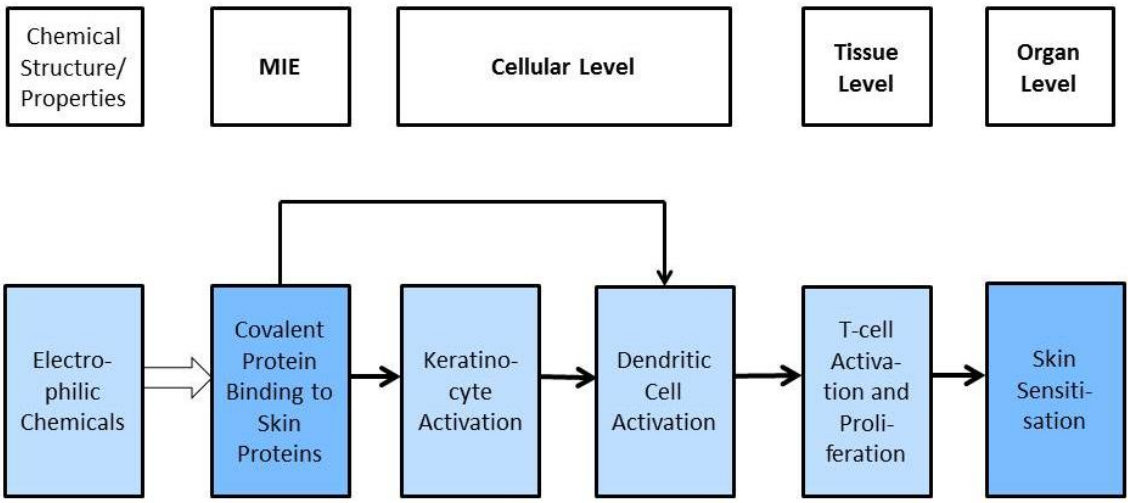

Figure 2. Example of an AOP associated with skin sensitisation (adapted from OECD 2012, ENV/JM/MONO(2012)10/PART1).

8. Members of the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), which assists international collaborative efforts in the area of AOPs, participated together with other scientists in the field in an international workshop in 2014 (http://www.saaop.org/workshops/somma.html). Resulting papers contributed to the refinement of conceptual aspects for the development and assessment of AOPs (Villeneuve et al., 2014a, b; Perkins et al., 2015; Garcia-Reyero, 2015; Becker et al., 2015; Groh et al., 2015a, b; Tollefsen et al., 2014). In one of the papers (Villeneuve et al., 2014a), five fundamental principles that guide AOP development are described:

(1) AOPs are not chemical specific;

(2) AOPs are modular and composed of reusable elements named key events (KEs) and key event relationships (KERs);

(3) an individual AOP, composed of a single sequence of KEs and KERs, is a pragmatic unit of AOP development and evaluation;

(4) networks composed of multiple AOPs that share common KEs and KERs are likely to be the functional unit of prediction for most real-world scenarios; and

(5) AOPs are living documents that will evolve over time as new knowledge is generated.

9. Another paper explored cases in WoE analysis as a basis for assessment of the maturity and level of confidence in AOPs based on modified Bradford-Hill considerations (Becker et al., 2015) that were included in the Users' Handbook. The major lessons learned from these experiences were also documented, and taken together with the case examples, contribute to better common understanding of the nature and form of documentation required to increase confidence in the application of AOPs.

10. Whilst AOPs may be depicted with a single axis (e.g. level of biological organisation; see Figure 1), toxicity is multi-dimensional (e.g., sex, species, age), so the pathway between a MIE and the final AO can vary significantly. This is especially true for more 'complex', longer-term endpoints, where effects are the result of multiple organ interactions (e.g., skin sensitisation), multiple events (e.g., repeated dose toxicity), accumulation over time (e.g., neural toxicity), or are related to a specific life stage of an organism (e.g. developmental toxicity). Nonetheless, although a number of biochemical steps are required for a toxic response to be realised, the MIEs are a prerequisite for all subsequent steps (Enoch and Cronin, 2010). With that said, it is understood that a single MIE may impact several signalling cascades (e.g. decreased protein expression of caspase-3, with concurrent activation of caspase-6), one or more of which may contribute to a specific AO. Additionally, an AOP is based on the fact that chemical interactions begin at the molecular level and not at the whole organism level. Thus, AOs observed *in vivo* are the results of a biological cascade initiated by the interaction of a chemical with endogenous biological molecules.

11. AOPs are a pragmatic simplification of biology, but it is recognised that they occur in a broader context that can be represented by AOP networks. A particular MIE may lead to several final outcomes and, conversely, several MIEs may lead to the same final outcome. However, an AOP should be designed to support an evaluation focusing on just one MIE and a single final AO.

12. Each component of an AOP may itself be influenced by other pathways ongoing within the biological system being modelled. Moreover, non-branched sequences of KEs that formulate an AOP unit likely possess KEs or KERs that are shared with other AOPs, generating interactions that lead to the creation of AOP networks. These systems of interacting AOPs, known as AOP networks, are thought to be more representative of most real-world scenarios (Villeneuve et al., 2014a; Knapen et al., 2015). AOPs can be visualised as a single linear sequence of KEs that leads to an AO, whereas AOP networks provide a broader picture and encompass a variety of available KEs and KERs that potentially can be perturbed leading to the manifestation of the same AO. These more complex networks may be viewed using other tools found in the AOP Knowledge Base (KB) (described below). These networks could be constructed to illustrate, for example, how an MIE in one organ (brain) could manifest as an AO in another (gonad).

13. OECD coordinates the efforts of building IT tools to enable the development and application of AOPs. The AOP-KB was launched on the 25th of September 2014 and consists of several modules. AOP-Wiki is an open-source interface that serves as central storage space of developed or under-construction AOPs that facilitates sharing of KEs and collaboration among AOP developers. AOP XPlorer is an additional module of the AOP-KB that assists in visualising AOP networks. The Effectopedia module captures additional structured information such as the quantitative response-response relationships between KEs, and the assays and biomarkers available for measuring the KEs. It displays this information via a graphical interface to facilitate the decision-making process. The fourth module, the Intermediate Effects database (IEDB), connects the AOP-KB to actual test results from in-vitro assays with prototypical chemicals, i.e. compounds used to underpin the scientific reasoning that leads to a certain AOP. The IEDB will be an IUCLID instance and will mostly use the OECD Harmonised Template (OHT) 201, which is geared towards non-classical test methods. OHT 201 data from third party IUCLID systems can be easily imported into the IEDB. The e.AOP.Portal is the main entry point for the AOP-KB, and provides a search mechanism for finding AOPs that are currently available in the AOP-Wiki and Effectopedia. The e.AOP.Portal hosts the status of all AOPs that are in the OECD work plan. All published AOPs are accessible through this same portal.

14. Development and release of AOPs and the AOP KB is expected to enable a variety of regulatory applications. For example, as part of the 2014 workshop described above, Groh et al. (2015a) explored how the AOP concept could be used to guide research aimed to improve our ability to predict AOs. In addition, using fish growth as a case study, they demonstrated how the AOP concept can be used to critically assess the knowledge available for specific chronic toxicity cases in order to identify existing

knowledge gaps and potential alternative tests (Groh et al., 2015b). Workshop groups also focused on elucidating the role of AOPs in informing the development of IATA for different regulatory purposes (Tollefsen et al., 2014), and explored further possible regulatory applications of AOPs depending on the relative degree of an AOP's development and scientific confidence (Perkins et al., 2015).

15. From the beginning it has been acknowledged that definitions and a checklist and/or evaluation framework is needed to help determine sufficiency for purpose. All AOPs at any level of development are useful; however, the extent to which the AOP can be relied upon in a specific regulatory context is related to its level of development, the level of uncertainty that can be tolerated and the level of evidence (e.g. detail, quality, and quantity of information and data) available (see Users' Handbook). The OECD works in conjunction with the EAGMST, which has primary responsibility for approving into the work plan a submitted proposal on a particular AOP, to assess adherence to guidance principles, completeness, and scientific robustness of AOPs as they are entered in the AOP-Wiki, and seeks member countries' approval. After EAGMST approval, the Working Group of the National Coordinators for the Test Guidelines (WNT) and the Working Party on Hazard Assessment (WPHA) are responsible for endorsement of AOPs and subsequent declassification from the Joint Meeting (JM).

16. The aim of this document is to provide the framework for consistent information gathering and organisation into an AOP, including a glossary of definitions for AOP-specific terminology. The document intends to provide insight into which pieces of information are necessary to develop an AOP and the accompanying terminology that should be used. Finally, the document also briefly outlines the potential use of AOPs in regulatory contexts. Guidance on how to use AOPs for integrated testing strategies is available (OECD, 2016b), whereas further guidance on their use for risk assessment will be developed in the future. More detailed guidance on AOP development and implementation in the AOP Wiki can be found in Users' Handbook, which is a supplement to the present document that will evolve over time based on the experience gained. The content of both documents, meaning the present guidance document and Users' Handbook, should be considered if developing or assessing an AOP.

DEVELOPMENT OF AN ADVERSE OUTCOME PATHWAY (AOP)

Identification of the Main Blocks of Information

17. The basic components of an AOP are the KEs, which are usually at different levels of biological organisation. These KEs are causally linked and essential to the AO under consideration, and they are measurable. MIEs and AOs represent specialized KEs within the context of a single AOP. The AOP is anchored at one end by an MIE, which represents the direct interaction of a chemical with a biological target, and at the other end by an AO, which can be at any biological level of organisation that is relevant to a regulatory decision. The second basic component of an AOP is the key event relationship (KER). A KER represents the connection between an upstream KE and the downstream KE in an AOP. Each of these pieces of information must be clearly identified and described during AOP development.

18. To develop an AOP, different types of data can be utilised including: structural alerts that reflect the types of chemicals that can initiate a pathway (i.e. with potential to interact with the molecular target that initiates the pathway), *in chemico* methods that measure the relative reactivity or chemical-biological interactions, *in vitro* assays that inform on the subsequent cellular responses (e.g. gene expression), *ex vivo* and *in vivo* mechanistic tests and, *in vivo* tests that measure the endpoint(s) that are directly relevant to the AO that drives regulatory decision-making (OECD 2011, ENV/JM(2011)6). It is worth repeating that AOPs are not chemical-specific and the pathway description should be independent from any specific chemical initiator. Nevertheless, in the context of a particular AO, experimental data derived from exposure to prototypic chemicals are useful for understanding the patterns of biological response. This information is used to identify KEs and KERs in the AOP and provide scientific evidence supporting the AOP. Thus, the AOP provides a scientific basis for linking effects in different dimensions (e.g. at different levels of biological organisation) to the final endpoint of the AOP (Figure 1).

Definition of the Molecular Initiating Event (at the Site of Action)

19. Chemical-induced perturbations of biological systems are at the molecular level. Most chemicals can interact with more than one molecular target. The MIE is a primary anchor or "the foundation" of the AOP; therefore, it is important to identify clearly the beginning of the cascade leading to the specified final AO relevant to the assessment, especially in cases where the MIE/AOP is being used to support *in silico* or *in chemico* predictions. Many MIEs are defined in the form of covalent binding to proteins and/or DNA. These types of MIEs are based on the principles of organic chemistry (i.e. electrophile-nucleophile reactivity). In contrast, 'receptor binding' or binding to enzymes are often based on non-covalent interaction, which are more selective in nature. Chemicals have different affinities for different targets. If internal exposure is sufficient to saturate a binding site on a receptor or enzyme, then the potency of activation or inhibition of an activity is what might drive toxicity. Understanding the MIE facilitates the identification and definition of the properties of chemicals most likely to induce the perturbation, such as bioavailability, structural requirements (especially for receptor binding) and metabolic transformation. Understanding the chemistry of potential inducers helps to define the molecular structure limitations for chemical category members acting in a similar manner.

20. In the ideal scenario, when the MIE is well-defined, not only should the potential of a chemical to elicit that event be recognised, but also the likely site of action should be noted. For example, metabolic transformation of a substance to an electrophilic species may be the same for skin sensitisation and liver fibrosis, but the site of action will be different (keratinocytes versus hepatocytes). For some endpoints, especially based on receptor binding mechanisms, the identification of the site of action is very important, as the 'conformation' and other properties of the receptor structurally define the type of molecules that can

bind to it. However, there are a number of AOs for which the identification of the site(s) of action of the MIE may be quite difficult (e.g. repeated dose) or have not been defined precisely (e.g. simple narcosis in fish). However, that does not necessarily mean the AOP, with ill-defined site(s) of action, is not useful.

Identification of the Adverse Outcome(s)

21. An AO is a specialised type of key event that is generally accepted as being of regulatory significance on the basis of correspondence to an established protection goal or equivalence to an apical endpoint in an accepted regulatory guideline toxicity test (OECD, 2016a). AOs can be defined based on a variety of dimensions (e.g., duration of exposure, sex, species, etc.). The final AO is notably an anchor of a particular AOP at the individual or population level. It is essential to clearly and precisely define the final AO relevant to regulatory decision-making (i.e., it corresponds to an accepted protection goal or common apical endpoint in an established regulatory guidance document). This helps to illustrate the mechanistic sequence of events leading to an AO that in the case of human health may constitute increased risk of a medical condition in a particular organ or organ system in an individual or in either the entire or a specified subset of the population; whereas, in ecosystems, this will most often be an outcome of demographic significance that has meaning in terms of estimates of wildlife population sustainability. Outcomes related to survival, fecundity or growth can be readily linked to population sustainability. However, many structural (e.g., gross abnormalities) and functional (e.g., behavioural anomalies) changes in organisms can have regulatory significance when combined with other factors and may add a valuable line of evidence in human and ecological risk assessment. At a minimum, an AOP should be linked to at least one AO of regulatory significance at the organ level of organisation or higher. Whenever feasible, the sequence of KERs should be extended to the population level in order to maximize the utility of the AOP for human health and ecological risk assessment (as appropriate to its applicability domain).

Identification of Key Events Leading to the Adverse Outcome

22. KEs are defined as steps along the pathway that represent intermediate events, typically at the different levels of biological organisation. To be a KE, the intermediate step must be essential to the AOP, and must be experimentally measurable. The Users' Handbook provides additional considerations and examples of the types of data that can be used to support the inclusion of a KE in an AOP.

23. The AOP includes the collection of KEs that lie between the final AO relevant to the assessment and the MIE. The relationship between adjacent events often can be defined in a manner that enables the inclusion of essential and measurable KEs that capture the main effects occurring along the pathway, rather than describing every single intermediate event. In an ideal scenario, the AOP should include a relatively small or minimal number of KEs required to establish the causal linkage/connection between the MIE (anchor 1) and the final AO (anchor 2). To provide support and data to evaluate an AOP, a range of *in vivo* and *in vitro* information on KEs and KERs, as well as information from high-throughput screening (HTS) assays, endpoints from high-content screening (HCS), omics approaches and even *in silico* methods can be used. As the number of KEs expands, the toxicological complexity becomes apparent.

24. Before the identification of KEs leading to AOs, an understanding of the normal physiological pathways is essential (e.g. reproductive processes, liver functions). This helps in the recognition of complex networks of processes that can be disrupted at the different levels of biological organisation. During the identification of KEs, a review of the existing literature is required to evaluate as much information as possible about the plausible mechanisms and intermediate steps leading to the final adverse effect. Judging the reliability and relevance of KE data in the available literature may include assessing critical parameters of the study design (e.g. exposure regime, duration of exposure, sampling times) for comparison and interpretation with respect to the final AO. While automated literature mining and evaluation tools including systematic review could aid to accelerate the development of an AOP, and

enhance objectivity and transparency of data evaluation, it is not required. The important point is that AOP development should be supported by the scientific literature and how that is accomplished should be up to those developing the AOPs, provided the primary considerations discussed in the Users' Handbook are addressed. Usually, multiple intermediate events are identified during the construction of a given AOP. Therefore, the assembled knowledge needs to be filtered to ensure it is appropriate for a particular AOP of interest. When a KE is present in more than a single AOP, the information can be shared between the AOPs.

Identification of the Key Event Relationships

25. All the KEs identified in an AOP are linked and defined in KERs. Describing the KERs in an AOP involves assembling and organising the types of information and evidence that define the scientific basis for inferring the probable change in, or state of, a downstream KE from the known or measured state of an upstream KE.

26. By convention, KERs may take one of two forms (OECD, 2016a). The pair of KEs linked via a KER may either be adjacent to one another in the sequence of KEs that define a given AOP. Alternatively, a KER may refer to a pair of KEs for which the relationship is thought to run through another KE (i.e., non-adjacent KEs in an AOP). It is not necessary to describe a KER for every possible binary pair of KEs that could be non-adjacent. However, the option to provide KER descriptions for non-adjacent KEs is particularly useful within the AOP Wiki, because empirical evidence supporting the linkages between adjacent KEs may not be available, or may be available only for KEs that are not directly adjacent. For example, some KE measurements may be fairly difficult to make, such that they are rarely made in routine studies. While there may be sufficient data to establish the KE as part of the AOP, much of the available WoE may ignore or "leap over" that particular KE. Including non-adjacent KEs descriptions allows the WoE for these relationships to be described and linked to other AOPs.

27. A critical component of AOP development is evaluating the WoE of the KERs. This includes assessment of biological plausibility of the relationships, and examination of the empirical evidence to support that the first KE occurs at doses equal to or lower than the subsequent KE, and at earlier time points than the downstream KE. More detailed information, including examples, is found below and in the Users' Handbook supplement.

AOP assessment

28. The evidence underlying a given AOP purposefully developed for possible use in regulatory purposes is evaluated based on the evolved Bradford Hill considerations, which are used for comparative analysis of WoE in the MOA context (Meek et al., 2014a; Meek et al., 2014b). However, they have been modified, as appropriate, to address the context for chemical agnostic AOPs and can be found in the Users' Handbook (OECD, 2016a).

29. The three primary considerations are:

1) the biological plausibility of KERs, which relies on an understanding of the fundamental biological processes involved and whether they are consistent with the causal relationship being proposed in an AOP;

2) the essentiality of KEs, which is considered in the context of an entire AOP and refers to experimental data for whether or not downstream KEs or the AO are prevented or modified if an upstream event is blocked (e.g. testing in knockout models or investigations of reversibility);

3) the empirical support of KERs, which is often based on toxicological data derived by one or more reference chemicals where dose-response and temporal concordance for the KE pair can be assessed.

30. When evaluating an AOP, the biological plausibility and empirical support are both evaluated for each KER separately, whereas essentiality is considered in the context of the AOP, based on supporting information for each of the KEs. This process not only supports sharing of components amongst AOPs as described earlier, but also helps to explicitly identify critical data gaps and uncertainties within the AOP as a basis to facilitate targeted research and evaluation for possible regulatory application. Once the WoE (composed of biological plausibility and empirical support) has been evaluated for each KER, and the essentiality has been evaluated for each KE, the evidence in support of the overall AOP can be summarised in a table following the instructions in the Users' Handbook (OECD, 2016a).

THE USES OF AOPS

31. A well described AOP with descriptive quantitative KERs that can be mathematically modelled would normally be required to support quantitation of hazard as a basis for risk assessment. However, even a well-defined qualitative AOP, with an accurately described sequence of events through the different levels of biological organisation, can provide valuable pieces of mechanistic information that can be used for many purposes (OECD 2011, ENV/JM(2011)6).

32. A variety of potential uses have been described for AOPs; the extent to which decisions can be supported by a given AOP depends on the level of uncertainty and quantitative understanding of the KERs. For example, by identifying and describing the KEs, AOPs can inform the work of the OECD Test Guideline Programme by describing the rationale for the use of particular methods and also by identifying potentially more predictive methods for development (further described below). AOPs can also be used as a basis for developing an IATA or an integrated testing strategy (ITS). They can also be used for further development and application of alternative approaches, such as read-across, where categories are first formed and data gaps filled within the category, leading to potential refinement, reduction and/or replacement of conventional *in vivo* testing.

33. AOPs can also be used to contribute to a number of regulatory contexts, including but not limited to: (1) priority setting for further testing, (2) hazard identification, (3) classification and labelling, and (4) risk assessment. As such, as one proceeds from (1) to (4), the level of uncertainty that can be tolerated decreases and the level of evidence (e.g. detail, quality, and quantity of information and data) presented in supporting the AOP increases.

34. A partially developed AOP (i.e. one where not all KEs are known) may be useful in priority setting for further testing and development. Similarly, partially developed AOPs may be used in hazard identification, as is currently performed with the OECD QSAR Toolbox. Physiologically-based pharmacokinetic (PBPK) modelling and toxicokinetics information on absorption, distribution, metabolism, and excretion (ADME) are not considered in AOP development, but will have to be addressed in application of AOPs in any of the regulatory contexts described above. AOPs can also serve as the starting point for MOA analysis for specific chemicals, incorporating consideration of chemical space and ADME.

35. A qualitative AOP is one where the KEs are supported by descriptions of how the KEs can be measured and KERs supported by empirical evidence in addition to plausibility or statistical inference, along with qualitative evaluation of the overall WoE supporting the AOP (Villeneuve et al., 2014a). In contrast, a quantitative AOP is based on the assembly of KEs supported by descriptions of how the KEs can be measured and the accuracy and precision with which the measurements are made along with KERs supported by quantitative understanding of what magnitude and/or duration of change in the upstream KE is needed to evoke some magnitude of change in the downstream KE (Villeneuve et al., 2014a).

36. During its development, an AOP is likely to begin as a putative AOP and evolve into a quantitative AOP as more information is accumulated, and generally, AOPs will exist along a continuum between putative, completely qualitative and completely quantitative. It is important to note that an AOP is never complete – it can continue to evolve, but that does not preclude its use to support decision-making. Potential uses for AOPs within OECD are described below.

Developing Chemical Categories and Further Development of the OECD QSAR Toolbox

37. One of the main target applications of AOPs is within the field of read-across and chemical category formation. AOPs can provide mechanistic information on the biological similarity of substances in grouping approaches when using the OECD QSAR Toolbox by addressing similarities of the MIE and KEs within one or multiple AOPs. In addition, AOPs can assist in the identification of refined testing strategies within a chemical category to address common mode of action of structurally similar substances, by focusing testing for specific KEs of a specific AOP (see also Use of AOPs within the context of Integrated Approaches to Testing and Assessment).

38. As demonstrated, for protein binding leading to skin sensitization in Version 3.0 of the OECD QSAR Toolbox, AOPs can be used to develop and refine chemical categories. In this example, three sets of information are collated and integrated: (1) a library of *in vivo* effects typically used in assessments (e.g. EC3 values in the local lymph node assay), (2) a library of MIEs (e.g. protein binding reaction), and (3) a library of intermediate events, typically data generated using *in vitro* methods (e.g. dendritic cell surface biomarkers) (OECD 2012, ENV/JM/MONO(2012)10/PART1; ENV/JM/MONO(2012)10/PART2.). Each endpoint can, in theory, be associated with a single or multiple chemical domain(s). With regard to chemical categories, the chemical structural space covered, or the applicability domain, depends on the chemicals assessed for the MIEs and the KEs within the AOP.

The Test Guideline Programme

39. By identifying and describing the KEs, the AOPs can inform the work of the Test Guideline Programme. Indeed, when the KEs are identified, one can propose the development of *in vitro* and *ex vivo* assays that detect direct chemical effects or responses at the cellular or higher levels of biological organisation, as well as screening assays for targets related to the MIEs identified (OECD 2011, ENV/JM/MONO(2011)8). Conversely, by linking proposals for the development of *in vitro* test methods to KEs in an AOP, the relationship to hazard endpoints relevant for regulatory purposes can be established.

40. For example, two methods identified in the AOP for protein binding leading to skin sensitisation have been proposed to OECD for test guidelines development: the Keratinosens assay (gene expression in human keratinocytes) (OECD, Test No. 442C and D) and the h-CLAT assay (cell surface marker (CD86) expression in human monocytic cells) (OECD, Test No. 442E).

41. However, a single AOP is unlikely to capture all events of potential regulatory relevance. AOP networks, which are based on AOPs that share at least one common element, potentially can provide a more realistic representation of pathways and networks of alterations leading to AOs. Analysis of these AOP networks can aid the prioritisation of assay development, whether the goal is to develop a single assay with predictive utility for multiple outcomes, or a battery of assays that are highly specific for predicting a specific endpoint of regulatory concern. For example, five AOPs related to reproductive and developmental toxicity for fish have been used to describe an AOP network and to illustrate how AOP networks can be used for assay development and refinement (Knapen et al., 2015).

Use of AOPs within the context of Integrated Approaches to Testing and Assessment (IATA)

42. An AOP can serve as a basis for developing an IATA or an ITS for any given endpoint. An AOP can assist in determining what additional information (and therefore, which test, if any) would increase the certainty of linking an initiating event and adverse effect(s). Moreover, a well-established AOP can be used for species-to-species extrapolation. The application of IATA and ITS may also lead to the refinement, reduction and/or replacement of conventional *in vivo* testing. There have been various approaches for assessing the scientific support and confidence underlying AOPs and their application in IATA have been

proposed (Tollefsen et al., 2014; Patlewicz et al., 2015; Perkins et al., 2015). A guidance document is available (OECD, 2016b) that outlines an approach for the use of the AOP concept in developing IATA, building upon the workshop held in 2014 on a framework for the development and use of IATA (OECD, 2015) and experience to date with the development of IATA.

SUMMARY

43. To implement a predictive strategy for risk assessment, results from *in vitro* toxicity assays focused on MIEs or cellular responses to MIEs will need to be extrapolated to effects on organisms and ultimately on populations. This can be achieved by developing AOPs that causally link MIEs with AOs. As these AOPs are intended to be used by regulatory agencies, this guidance provides an introduction to the development and assessment of AOPs, aiming to standardise the way in which AOPs are developed, documented and reviewed. Following their development and review, endorsed AOPs are published in the OECD Series on Adverse Outcome Pathways. However, because scientific knowledge progresses, the publication of an AOP in this series does not preclude further updates or new contributions to that AOP.

44. AOPs should provide a transparent, mechanistically-based framework for developing or refining chemical hazard categories, as well as proposing and prioritising targeted *in vitro* and *in vivo* testing. By understanding the likelihood of effects at lower levels of biological organisation from structure-activity relationships (SARs) and *in chemico* and *in vitro* assays, one could efficiently determine if additional tests at higher levels of biological organisation (e.g. *in vivo* assays) are required (Meek et al., 2011). The guidance provided here, along with incorporation of evolving MOA analysis presented in the MOA Framework (updated by WHO/IPCS in 2012; Meek et al. 2014a) and found in the Users' Handbook, will assist in incorporating mechanistic data and computational models in the decision-making process.

45. As indicated by Bauch et al., not all KEs in an AOP must be fully described in order to be used in an assessment (Bauch et al., 2011). Use of an AOP or network of AOPs for a particular purpose will involve consideration of the information concerning the MIE and the KEs that lead to the final AO, which is the basis of the AOP assessment, as well as the WoE for each KER and the overall AOP. What is considered sufficient knowledge of an AOP can be use-dependent, with a greater knowledge and/or confidence required for applications with greater potential impact (Meek et al., 2011).

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ANNEX I: GLOSSARY OF TERMS RELATED TO ADVERSE OUTCOME PATHWAYS

The terms appearing in this guidance document are organised in alphabetic order. Some of the terms below have been described in a variety of sources, with largely overlapping definitions. However, the most evolved and complete definition for these terms is included here for consideration.

ADME

An acronym in pharmacokinetics/toxicokinetics and pharmacology/toxicology for absorption, distribution, metabolism, and excretion; describes the disposition of a pharmaceutical/chemical compound within an organism. The four processes all influence the drug/chemical levels and kinetics of drug exposure to the tissues and hence influence the performance and pharmacological/toxicological activity of the compound (Pharmacology Study Guide, 2007).

Adverse outcome

An Adverse Outcome is a specialised type of key event that is generally accepted as being of regulatory significance on the basis of correspondence to an established protection goal or equivalence to an apical endpoint in an accepted regulatory guideline toxicity test (OECD, 2016a).

Note: Depending on whether the protection goal is for human health or ecological health, the endpoints considered may differ.

Adverse Outcome Pathway (AOP)

Conceptually, an AOP can be viewed as a sequence of events commencing with initial interactions of a stressor with a biomolecule in a target cell or tissue (i.e., molecular initiating event), progressing through a dependent series of intermediate events and culminating with an adverse outcome. AOPs are typically represented sequentially, moving from one key event to another, as compensatory mechanisms and feedback loops are overcome (OECD, 2016a).

Apical endpoint

Apical endpoints are empirically verifiable outcomes of exposure, such as death, developmental anomalies, breeding behaviors, impaired reproduction, physical changes and alterations in the size and histopathology of organs, including clinical signs or pathologic states, that are indicative of a disease state (Krewski et al., 2011; Villeneuve and Garcia-Reyero, 2011).

Note: Endpoints (outcomes) considered to be apical may differ if used as a surrogate for human health versus ecological health.

Cellular response

The binding of a chemical signals to the corresponding receptors and induces events within the cell that ultimately change its behaviour. The nature of these intracellular events differs according to the type of receptor. Also, the same chemical signal can trigger different responses in different cell types (http://global.britannica.com/EBchecked/topic/101396/cell/37445/Cellular-response).

Chemical category

A group of chemicals whose physico-chemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity (or other similarity characteristic) (OECD, 2007).

Effectopedia

Effectopedia is a collaborative research platform for AOP development, modelling and use. The knowledge (qualitative and quantitative) is organized in nested layers. The most abstract layer of pathway representation is a visual diagram of the pathway structure. The next level of details is captured in custom-built interfaces for the individual elements (chemicals, effects, links, test methods, *in silico* models, etc.) and provides uniform representation of structured summary information. The overall goal of Effectopedia is to aggregate, in a single source, all information needed to allow the development of quantitative AOPs and their use in regulatory decision-making context (Aladjov, personal communication).

Endpoint

The recorded observation coming from an *in chemico* method, an *in vitro* assay or an *in vivo* assay (OECD, 2011).

Integrated Approaches to Testing and Assessment (IATA)

An Integrated Approach to Testing and Assessment is an approach based on multiple information sources used for hazard identification, hazard characterisation and/or safety assessment of chemicals. An IATA integrates and weights all relevant existing evidence and guides the targeted generation of new data, where required, to inform regulatory decision-making regarding potential hazard and/or risk. Within an IATA, data from various information sources (i.e. physicochemical properties, *in silico* models, grouping and read-across approaches, *in vitro* methods, *in vivo* tests and human data) are evaluated and integrated to draw conclusions on the hazard and/or risk of chemicals. Within this process, the incorporation of data generated with non-animal testing and non-testing methods is expected to contribute considerably to a reduction of testing in animals (OECD, 2016b). The output of an IATA is a conclusion that, along with other considerations, informs regulatory decision-making (OECD, 2016b).

Integrated Testing Strategy (ITS)

A defined approach to testing and assessment can be designed in different ways, and may take for example the form of a Sequential Testing Strategy (STS) or an Integrated Testing Strategy (ITS). An ITS is an approach in which multiple sources of data or information are assessed at the same time by applying a variety of specific methodologies to convert inputs from the different information sources into a prediction. For this purpose, a variety of specific methodologies can be applied, such as statistical and mathematical models (OECD, 2016b).

Key Event (KE)

A key event is a change in biological state that is both measurable and essential to the progression of a defined biological perturbation leading to a specific adverse outcome (OECD, 2016a).

Key event relationship (KER)

A key event relationship is a scientifically-based relationship that connects one key event to another, defines a directed relationship between the two (i.e. identifies one as upstream and the other as downstream), and facilitates inference or extrapolation of the state of the downstream key event from the known, measured, or predicted state of the upstream key event (OECD, 2016a).

Levels of biological organisation

Atom, molecule, cell, tissue, organ, organ system, organism (individual), population, community (see Figure 1) (Villeneuve and Garcia-Reyero, 2011).

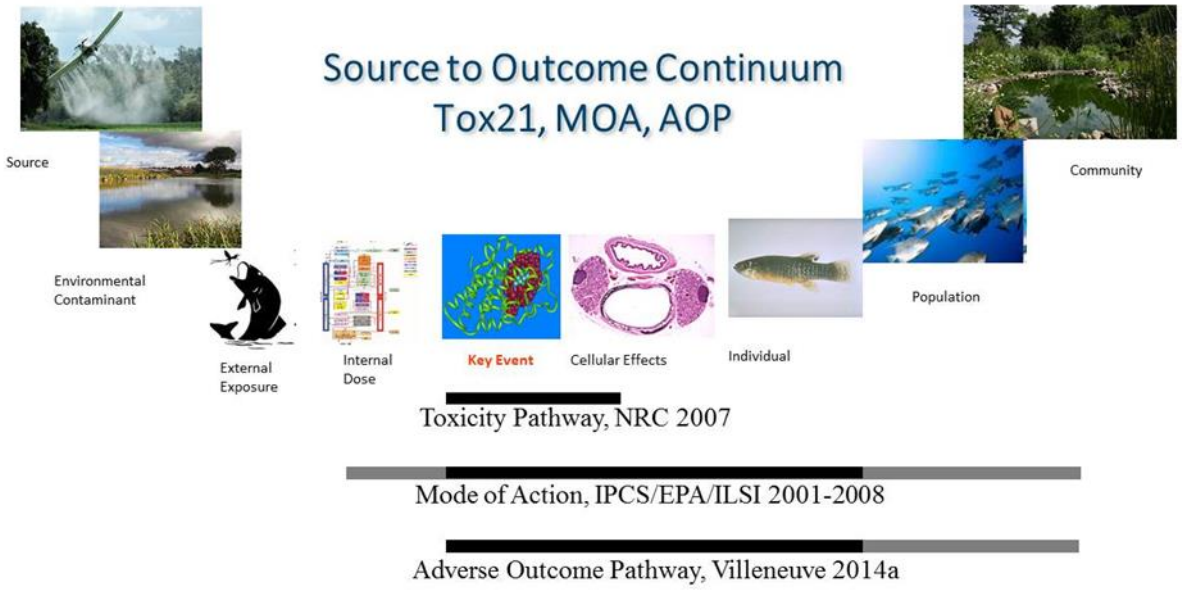

Figure 1. Representation of the relationships between Toxicity Pathways, Mode of Action Pathways and Adverse Outcome Pathways. The black bars represent the breadth of research common to these concepts. The grey bars represent the theoretical extent of the concepts (adapted from OECD 2011).

Mechanism of action

Mechanism of action for toxicity is the detailed molecular description of key events in the induction of cancer or other health endpoints. Mechanism of action represents a more detailed understanding and description of events than is meant by mode of action (North American Free Trade Agreement NAFTA, 2011).

Mode of action (MOA)

Mode of action is defined by WHO as "A biologically plausible sequence of KEs leading to an observed effect supported by robust experimental observations and mechanistic data. A mode of action describes key cytological and biochemical events – that is, those that are both measurable and necessary to the observed effect – in a logical framework." World Health Organization (2009) Environmental Health Criteria 240: Principles and Methods for the Risk Assessment of Chemicals in Food. WHO, Geneva, (Definitions page A-25) http://www.who.int/foodsafety/publications/chemical-food/en/.

Molecular Initiating Event (MIE)

A molecular initiating event is a specialised type of key event that represents the initial point of chemical interaction at the molecular level within the organism that results in a perturbation that starts the AOP (OECD, 2016a).

Molecular screening

Molecular screening combines rapid screening methods and toxicogenomics with the objective of applying biochemical and cellular genomic methods to category analysis. The premise of molecular toxicity screening is driven by interactions of chemicals with cellular targets of one form or another. To initially assess toxicity, one must identify the proper target of concern and an appropriate assay is needed to assess the likelihood of interaction with the chemical(s) of concern (OECD, 2008).

Pathway perturbation

Critical alteration of a toxicity pathway by an environmental agent or its metabolites that can impair normal biological function to such an extent that an adverse health effect may occur (Krewski et al., 2011).

Site of action

The site of action can be the biological molecule that interacts with a chemical, or can refer to a more specific site on the macromolecule of interest, such as the ligand binding domain of a receptor. The site of action also can be viewed in the context of the particular cell or tissue type in which the molecular initiating event takes place (Schultz, personal communication). Note that site of action may be species-specific.

Structural alerts

Structural alerts are atom-based fragments which, when present in a molecule, are an indication that a compound can be placed into a particular category (Schultz, 2010).

Systems biology

Systems biology is defined as the biology of dynamic interacting networks. It is a holistic approach in the biomedical sciences aimed at deciphering the complexity of biological systems by starting from the understanding that the networks that form the whole of living organisms are more than the sum of their parts. Systems biology integrates approaches from biology, computer science, engineering, bioinformatics, physics and other disciplines to predict how these systems and networks change over time and under varying conditions, including chemical exposures. It requires the use of a variety of analytical platforms as well as bioinformatics, data integration, and modelling (Jain, 2010).

Note: Systems biology involves (1) collection of large sets of experimental data (by high-throughput technologies and/or by mining the literature of reductionist molecular biology and biochemistry), (2) proposal of mathematical models that might account for at least some significant aspects of this data set, (3) accurate computer solution of the mathematical equations to obtain numerical predictions, and (4) assessment of the quality of the model by comparing numerical simulations with the experimental data (Duffus et al., 2007).

Toxicity Pathway

Cellular response pathways that, when sufficiently perturbed, are expected to result in adverse health effects are termed *toxicity pathways* (NRC, 2007) (defined in the context of Figure 1). A toxicity pathway relates to the perturbation of a normal biochemical pathway from the molecular initiating event to the cellular effect. Although it is at the heart of the mode of action and AOP concepts, it is not linked directly to an apical effect.

Weight of evidence (WoE)

WoE is a comprehensive, integrated, often qualitative judgment of the extent and quality of information supporting an hypothesis for which the approaches and tools vary, depending on the context (Weed, 2005; WHO-UNEP, 2012). For AOPs, the WoE is addressed based on a specified subset of

considerations modified from those proposed by Bradford Hill (B/H) for assessment of causality in epidemiological studies (Hill, 1965), drawing on previous experience in mode of action analysis. Defining questions and the nature of supporting data for each of the relevant considerations is included in Annex 1 of the Users' Handbook to the OECD Guidance on AOPs (OECD, 2016a).

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