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## Guidance on Biological Relevance

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### Abstract

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## Summary

EFSA requested its Scientific Committee to prepare a guidance document providing generic issues and criteria to consider biological relevance, particularly when deciding on whether an observed effect is of biological relevance, i.e. is adverse (or shows a positive health effect) or not.

The opinion clarifies a number of definitions and concepts, such as, responses of a biological system to exposure, mode of action and adverse outcome pathways, thresholds, critical effect, modelling approaches, biomarkers, which are central to biological relevance and in order to achieve that these concepts are used in a consistent way across EFSA areas of activity.

The list of generic issues (e.g. nature and size of the biological changes or differences, including the relevance of the biological systems where the effects are observed) to consider when deciding on whether an observed effect is biologically relevant should be applicable to all relevant EFSA Scientific Panels and Scientific Committee.

Several case studies covering the various EFSA areas are referred to in the guidance and annexed to the opinion to illustrate the proposed approach.

A framework was developed in which biological relevance is considered at three main stages related to the process of dealing with evidence:

- Development of the assessment strategy, in this context, specification of agents, effects, subjects and conditions.
- Collection and extraction of data, i.e. identification of potentially biologically relevant evidence/data as specified in the Assessment strategy
- Appraisal of the relevance of the agents, subjects, effects and conditions, i.e. reviewing dimensions of biological relevance for each data set.
  - The agent; it should be considered whether the assessment is based on the agent of concern or on a surrogate agent.
  - The subject; in case proxies are used consider the relevance of effects occurring in these for the subject under assessment.
  - The effect; a wide variety of effects may be considered. Consideration should be given as to whether the effect is causally related to exposure to the agent, and the nature of the effect should also be taken into account, i.e. homeostatic response, adaptive, directly or indirectly adverse or beneficial. Finally for effects where the size of the effect is critical, it should be assessed whether the magnitude of the effect is sufficient to be of biological relevance and thereby of importance for the assessment outcome. It should be noted that the biological relevance of an effect can vary according to the assessment question.
  - The conditions; it should be considered whether the conditions of a biological (test) system, e.g. exposures, models, are relevant for the

41 In standardised assessments, i.e. assessments that strictly follow guidelines or  
42 guidance documents, the assessment questions are generally already defined in  
43 a standard form, and there is also a standard procedure for assessing them.  
44 Sometimes also regulations prescribe what kinds of data are needed for the  
45 assessment. In these cases biological relevance of effects, and biological systems  
46 may be predefined.

47 Each step of relevance considerations may be source of uncertainty. The  
48 assessor should address these uncertainties as a part of the general uncertainty  
49 analysis of the assessment. The SC Guidance on Uncertainty (EFSA 2016b)  
50 should be followed.

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## 1. Introduction

### 1.1. Background and Terms of Reference as provided by EFSA

As per EFSA's Founding Regulation (EC) No 178/2002 of the European Parliament and of the Council, "the EFSA Scientific Committee shall be responsible for the general coordination necessary to ensure the consistency of the scientific opinion procedure, in particular with regard to the adoption of working procedures and harmonisation of working methods". The EFSA Science Strategy 2012-2016 echoes this key responsibility of the Scientific Committee by setting the development and harmonisation of methodologies and approaches to assess risks associated with the food chain as one of the four strategic objectives for EFSA.

The recent opinion of the Scientific Committee (SC) on "Priority topics for the development of risk assessment guidance by EFSA's Scientific Committee" (EFSA, 2013) gives recommendations for the preparation of new or revision of existing guidance documents. The criteria for prioritising guidance documents to be developed are:

- Across Panel Relevance
- Critical importance including urgency of topic to be addressed for several Panels
- Topic not being addressed by an individual Panel
- Sufficient information available to develop meaningful guidance
- International dimension.

The development of guidance on biological relevance was identified by the EFSA Scientific Committee as one of the three high priority topics for 2014.

In the EFSA opinion on the hazard assessment of endocrine disruptors (EFSA Scientific Committee, 2013), the concept of biological relevance assumes that a "normal" biological state can be defined and the definition of normality is closely linked to adversity of an effect observed during toxicity testing or in epidemiological studies. Distinguishing adverse effects from physiological adaptive effects is not only crucial in identifying a No Observed Adverse Effect Level (NOAEL) from experimental toxicity studies but also when using the benchmark dose (BMD) approach as recommended by the SC (EFSA Scientific Committee, 2009).

In its opinion on biological relevance versus statistical significance, the EFSA Scientific Committee gave a wider definition of biological relevance than just a modification of a physiological system, making it more applicable to the various EFSA working areas. In that opinion, biological relevant effect is defined as an effect considered by expert judgement as important and meaningful enough for human, animal, plant or environmental health. It implies a change that may alter how decisions for a specific problem are taken (EFSA Scientific Committee, 2011).

The above definition implies that guidance is provided to the various EFSA panels on what "harm" means, and to define a number of related concepts such as "effect size". When a particular risk assessment considers several effects, the overall picture, using a multivariate approach, should be considered to decide

whether the available body of knowledge allows to conclude on an effect to be adverse or not. Given the broad remit of activity of EFSA, the purpose of this self-task mandate is to provide the Scientific Panels with generic issues to consider when discussing on biological relevance, i.e. being adverse (or showing a positive health effect) or not.

#### Terms of reference

EFSA requires its Scientific Committee to prepare a guidance document providing generic issues and criteria to consider when deciding on whether an observed effect is of biological relevance, i.e. is adverse (or shows a positive health effect) or not.

The opinion should clarify a number of definitions and concepts, such as, adverse, adaptive, harm, homeostasis, biological threshold in order to achieve that these concepts are used in a consistent way across EFSA areas of activity.

The list of criteria / generic issues (e.g. nature and size of the biological changes or differences) to consider to decide whether an observed effect is biologically relevant should be applicable to all relevant EFSA Scientific Panels and Scientific Committee.

Several case studies covering the various EFSA areas will be annexed to the opinion to illustrate the proposed approach.

Links should be established with related ongoing EFSA activities, particularly with the SC working group on weight of evidence, the activity of the Assessment and Methodological Support (AMU) Unit on promoting methods for evidence use in scientific assessments (PROMETHEUS), and the SC working group on uncertainty in risk assessment. Relevant international activities and developments in the area, such as the IPCS/WHO mode of action framework should also be considered.

In view of the horizontal aspect of this topic and the need to get a common agreement and understanding of what biological relevance means, representatives of EFSA sister agencies, EC non-food committees and international bodies (e.g. WHO) should be invited to participate in the working group.

### **1.2. Interpretation of the Terms of Reference**

When addressing the mandate, the Scientific Committee acknowledged that the issue of biological relevance in risk assessment has a broader meaning than biologically relevant effect as described in the Terms of Reference. In fact, it encompasses also aspects related to the definition of the problem formulation. This, in turn, guides the development of the assessment strategy, which includes the decision on which data to use for the assessment (relevance of the data).

Aspects related to the reliability of the various pieces of evidence used in the assessment are outside the scope of this mandate, as they are the subject of another SC guidance on weight of evidence (under development).

The purpose of this document is to discuss and provide guidance across Panels/Units of EFSA on the above mentioned issues and how they should be addressed during the risk assessment process. Although these issues are expected to be considered consistently by the different Panels/Units, their application will always rely on expert judgement.



### 1.3. Relation to other relevant EFSA guidance documents

The guidance on the use of the weight of evidence (under development) builds on the conceptual approach for scientific assessments as described in PROMETHEUS (EFSA, 2015a), which describes the overall process for dealing with data and evidence. The process has four steps as shown in figure 1:

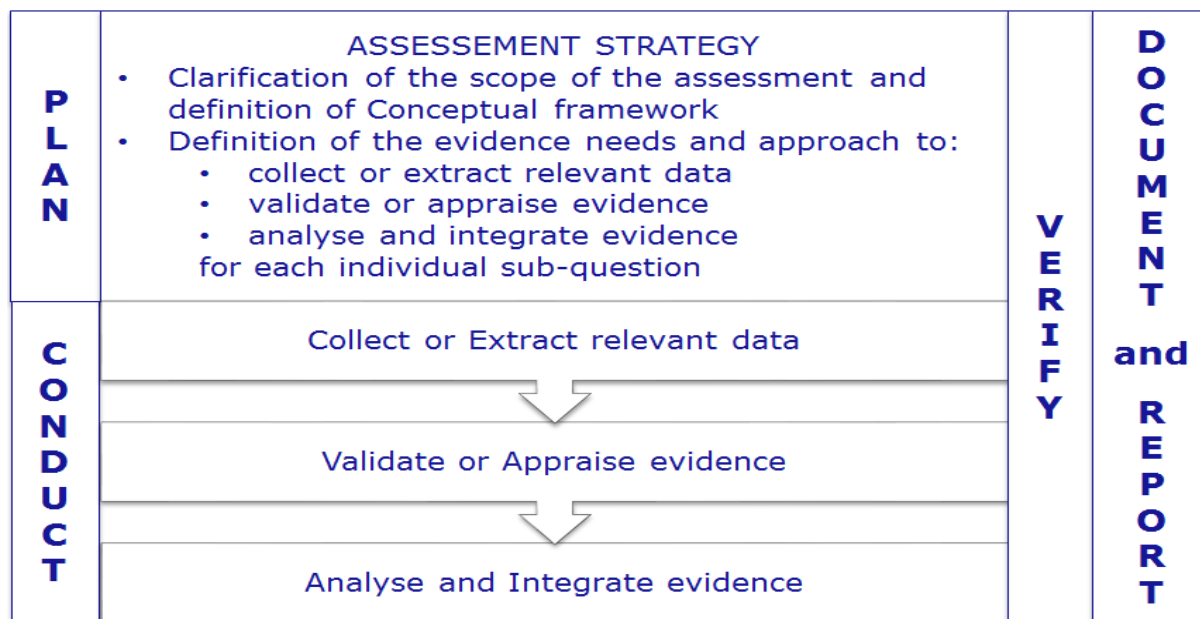


Figure 1: The process for dealing with data and evidence when conducting an assessment (EFSA, 2015a)

Transparent reporting of all assumptions and methods used, including expert judgement, is necessary to ensure that the assessment process leading to the conclusions is fully comprehensible.

‘Open EFSA’ aspires both to improve the overall quality of the available information and data used for its scientific outputs and to comply with normative and societal expectations of openness and transparency (EFSA, 2009, EFSA 2014). In line with this, EFSA is publishing three separate but closely related guidance documents to guide its expert Panels for use in their scientific assessments (EFSA, 2015a). These documents address three key elements of the scientific assessment: the analyses of Uncertainty, Weight of Evidence and Biological Relevance.

The first document provides guidance on how to identify, characterise, document and explain all types of uncertainty arising within an individual assessment for all areas of EFSA’s remit. The Guidance does not prescribe which specific methods should be used from the toolbox but rather provides a harmonised and flexible framework within which different described qualitative and quantitative methods may be selected according to the needs of each assessment.

The second document on weight of evidence provides a general framework for considering and documenting the approach used to evaluate and weigh the assembled evidence when answering the main question of each scientific assessment or questions that need to be answered in order to provide, in



conjunction, an overall answer. This includes assessing the relevance, reliability and consistency of the evidence. The document further indicates the types of qualitative and quantitative methods that can be used to weigh and integrate evidence and points to where details of the listed individual methods can be found. The weight of evidence approach carries elements of uncertainty analysis: that part of uncertainty which is addressed by weight of evidence analysis does not need to be reanalysed in the overall uncertainty analysis, but may be added to.

This document provides a general framework to addresses the question of biological relevance at various stages of the assessment: the collection, identification and appraisal of relevant data for the specific assessment question to be answered. It identifies generic issues related to biological relevance in the appraisal of pieces of evidence, in particular, and specific criteria to consider when deciding on whether or not an observed effect is biologically relevant, i.e. adverse (or shows a positive health effect). A decision tree is developed to aid the collection, identification and appraisal of relevant data for the specific assessment question to be answered. The reliability of the various pieces of evidence used and how they should be integrated with other pieces of evidence is considered by the weight of evidence guidance document (under development).

EFSA will continue to strengthen links between the three distinct but related topics to ensure the transparency and consistency of its various scientific outputs while keeping them fit for purpose.

#### 1.4. Audience and degree of obligation

This Guidance is aimed at all those contributing to EFSA assessments and provides a harmonised, but flexible framework to determine biological relevance that is applicable to all areas of EFSA's work and all types of scientific assessment. In line with improving transparency (EFSA, 2006, EFSA, 2009) and reporting (EFSA 2014b, EFSA 2015a), the Scientific Committee considers the application of this guidance to be unconditional for EFSA. Each assessment must clearly and unambiguously document:

- what evidence was considered;
- how the evidence was weighed and integrated in terms of relevance;

The document provides guidance on the general principles to determine the biological relevance but assessors have the flexibility to choose the degree of refinement in applying them. The Scientific Committee considers that these should be fit for the purpose of the scientific assessment.

## 2. Data and methodologies

The process for dealing with data and evidence in an assessment as defined in the PROMETHEUS project deliverable 1 (EFSA 2015a) was used as a framework for developing a guidance on biological relevance. A fundamental step in this process is represented by planning a strategy for the assessment including:

- the problem formulation;
- the conceptual framework;
- the definition of the evidence needs; and
- the approach for:
  - Collecting or extracting relevant data;
  - Validating or appraising evidence;
  - Analysing and integrating evidence.

In line with the “Open EFSA” objective (i) to improve the overall quality of available information and data used for its outputs and (ii) to comply with normative and societal expectations of openness (EFSA, 2014b, 2015a), a targeted consultation of national and international scientific advisory bodies was organised on an EFSA Journal editorial presenting “Increasing robustness, transparency and openness of scientific assessments” and a document providing the individual background and terms of reference of four related activities:

- the PROMETHEUS (“PROMoting METHods for Evidence Use in Scientific assessments”) project which aims to further improve the methods for “dealing with data and evidence” (i.e. collecting/extracting, validating/appraising, analysing and integrating data and evidence) in EFSA scientific assessments and to increase their consistency.
- Three topics for guidance developments: (i) the identification of biological relevance of adverse/positive health effects from experimental animal and human studies; (ii) The use of the weight of evidence in scientific assessments; (iii) The characterisation of uncertainties in scientific assessment.

A workshop was then organised on 29 and 30 June 2015 in Brussels to consult with national and international bodies including European Agencies, EC Scientific Committees, national agencies and international bodies with an interest in biological relevance. One objective of the workshop was to present the terms of reference of the two SC working groups on weight of evidence and biological relevance, clarify the objectives and the scope of the resulting guidance documents, and capture from the audience relevant work that should be considered by the working groups when drafting the guidance.

A Working Group composed of Panel Experts and EFSA Staff representing all EFSA areas of activity was created to address the above mandate. Members of the working group were first asked to describe in short documents how biological relevance was considered in past assessments (see Annexes). Key concepts and definitions that came out of these examples or that came out from the above consultations and that one should have in mind when considering the relevance of a dataset or a piece of evidence for an assessment, have been organised into a conceptual framework and are further described in the following sections.

### 3. Assessment

Relevance is a fundamental concept, which is considered during three steps in the process of dealing with evidence as described in PROMETHEUS (Promoting Methods for Evidence Use in Scientific Assessments) (EFSA, 2015a):

- Development of the assessment strategy
- Collection and extraction of data
- Appraisal of the evidence.

Assessing relevance in each of these steps has different implications in terms of the elements to be considered and their impact on the conclusions.

The assessment strategy should specify which scientific evidence (data) would be relevant for answering the assessment question(s). However, relevance can only be determined if the question(s) for assessment is well-defined. It is therefore important to ensure a clear understanding and interpretation of the question(s) for assessment between the risk assessors and with the risk manager before developing the assessment strategy.

Having clarity on the assessment question(s) would provide guidance on what data is relevant or irrelevant. Irrelevance is a practically useful concept, because it identifies what can be excluded from the assessment: which effects, which data, which model, which parameters, etc. and hence what to include. Note that if an effect has relevance, then all studies testing for that particular effect become relevant, as both positive and negative findings may influence the answer to the assessment question.

When the relevant data have been collected the biological relevance of the effects should then be appraised.

It is important to note that the reliability of the various pieces of evidences used and how they should be integrated with other pieces of evidence in the assessment are outside the scope of this SC guidance on relevance, as these are the subject of another SC guidance on weight of evidence (under development).

In the following text some fundamental concepts related to biological relevance will be presented following a framework for consideration of relevance. This includes considerations to be done at the three steps:

- Development of the assessment strategy, in this context, specification of agents, effects, subjects and conditions.
- Collection and extraction of data, i.e. identification of potentially biologically relevant evidence/data as specified in the Assessment strategy.
- Appraisal of the relevance of the agents, subjects, effects and conditions, i.e. reviewing dimensions of biological relevance for each data set.

### 3.1. Concepts about biological relevance

In 2011 the Scientific Committee expressed an opinion addressing the concept of Statistical Significance and Biological Relevance in the context of assessing scientific evidence. The following definition of biological relevance was developed:

*A biologically relevant effect can be defined as "an effect considered by expert judgement as important and meaningful for human, animal, plant or environmental health. It therefore implies a change that may alter how decisions for a specific problem are taken" (EFSA, 2011).*

The guidance, in which this definition of Biological Relevance was developed; stressed that a statistically significant effect should not automatically be considered relevant for the outcome of an assessment, but that an independent evaluation of the effects as to its relevance was required. In this context the definition implies that all effects that directly or indirectly would have the potential to influence the outcome of the assessment should be considered. The definition also implies that an effect that has no possibility or potential of altering how decisions are made or in other words would have no direct or indirect impact on the outcome of an assessment, should be considered as irrelevant.

#### 3.1.1. About responses of a biological system to exposure

A biological system usually reacts to signals from its environment, including the agent (e.g. nutrient, substance, microorganism, pathogen or invasive species) under assessment. The quality of the response of the biological system, hereafter called the nature of the effect, can be either adaptive, adverse or beneficial and may occur at different levels, e.g. molecular cell, organ, individual, population or ecosystem.

An effect is considered "adverse" when leading to a *change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity to compensate for additional stress or an increase in susceptibility to other influences" (WHO, 2009).*

An adverse effect might be primary (directly induced by the agent) or secondary (e.g. related to stress or nutritional imbalance induced by the agent) (Lewis, 2002). It is important to distinguish this for the interpretation of the effect in the context of the assessment question.

Adverse does not necessarily mean irreversible. An adverse change might be reversible. Whether adverse findings are reversible can be evaluated in an animal test model if animals that are allowed to recover after an appropriate non-dosing period. For example, adverse changes in regenerating tissues can recover (effects on spermatogenesis can lead to the non-function of the genital system and lack of the possibility to reproduce; but recovery can happen after a withdrawal of the exposure) (Perry et al, 2013).

In environmental risk assessment, the concept of environmental harm is used, which is defined as the measurable adverse change in a natural resource or the

measurable impairment of a natural resource service. It may occur directly or indirectly (EC, 2004), or as a measurable (or otherwise observable) loss or damage that has adverse (and significant) impact upon conservation and sustainable use of biodiversity (CBD, 2009).

The concept of “recovery” is also used in environmental risk assessment: the return of the perturbed (ecological) endpoint (e.g. species composition, population density) to the window of natural variability as observed in the undisturbed state of the (eco)system of concern (e.g. before the stressor event took place), or to the level that is not significantly different anymore from that in control or reference systems. It should be noted that a system that has been subject to an adaptive response or to recovery might not necessarily return to the same stable state that it exhibited before the disturbance. (EFSA, 2016)

When subject to a disturbance, a biological system enters in a transient state: a process variable has been changed and the system has not yet reached steady state. Some systems have the capacity to regulate their internal environment and to maintain a stable, relatively constant condition of properties; it is called “homeostatic capacity”. Resilience represents the amount of disturbance that can be absorbed by a system before the system changes or loses its normal function, or the time taken to return to a stable state, within the normal operation range following the disturbance (Gunderson, 2000).

The response to exposure to an agent can be “adaptive”, i.e. involving a process whereby a cell or organism respond to an agent so that the cell or organism will survive in the new environment that contains the agent without impairment of function (Keller et al 2012). One type of adaptive response is the homeostatic response, which is an active regulation of a parameter to keep it within its physiological range (e.g. glycaemic regulation, body temperature regulation). Another type of adaptive response can occur outside physiological boundaries and may be detrimental to health; therefore, it requires further considerations as to its adversity (e.g. composition of gut microbiota, liver enzyme induction). This issue is also discussed in Annex I, regarding chemicals that may affect thyroid hormone regulation).

An effect is considered “beneficial” if it has the probability to be linked to a positive (health) effect and/or the probability to be linked to a reduction of an adverse health effect in an organism, system or (sub)population, in reaction to exposure to an agent (EFSA, 2016c). The relevance of biological outcomes in terms of benefits follows similar rules as those for adverse outcomes. Yet, it should be noted that for adverse outcomes, very often data from animal test systems or in vitro studies are used, whereas for (health) benefits, studies on the target species and population group are required, e.g. studies in humans in the case of health claims for food (see Annex G). In benefit assessment, EFSA is normally with the exception of certain agricultural products and processes (e.g. growth promotion of animals) not considering economic aspects. Such benefits may not necessarily be beneficial for the health of the target species (see Annex E).

### 3.1.2. About Mode of Action and Adverse Outcome Pathway

When an agent (e.g. chemical) causes a toxic adverse effect in an organism, the effect is often a result of a sequence of events starting with a molecular interaction between the agent and the organism. To what extent a molecular effect should be considered biologically relevant depends on whether and how



close it might be linked to an adverse outcome, either as a key event or indirectly having an impact on a key event in the in the sequence leading to an adverse outcome. Mode of Action (MoA) and Adverse Outcome Pathway (AOP) are concepts used in this context. In many cases also the magnitude of the effect might be critical for the determination of its biological relevance.

The definition of Mode of action (MoA) has evolved over time and derives from earlier works by the US-EPA (US EPA, 1986, 2005) and the WHO. MoA analyses have been applied to a number of case studies for non-genotoxic and genotoxic chemicals (WHO, 2006a,b). The current WHO definition for MoA is 'a biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data'. MoA describes key cytological and biochemical events – that is, those that are both measurable and necessary to obtain the observed effect – in a logical framework (Boobis et al., 2006; WHO, 2009; Meek et al., 2014). In the US, MoA has been used as a term to reference a mechanistic understanding of the impact of a chemical on human health and to reference other terms from epidemiology including 'disease signature' and 'network perturbations'. Toxicologists would also refer to the same concept using the terms 'toxicity pathway, MoA, adverse outcome pathway or mechanism of action' as used by the National Research Council (NRC) report, Science and Decisions: Advancing Risk Assessment (2009) (NRC, 2009) and the Nextgen report of the US-EPA (US-EPA, 2013). Modified Bradford Hill criteria can be used to analyse the biological plausibility of key events and the weight of the related evidence. Mechanism of action is defined as the specific biochemical interaction through which a substance produces an effect on a living organism or in a biochemical system (WHO/IPCS EHC 240). MoA does not imply full understanding of mechanism of action, which refers to a detailed molecular description of individual biochemical and physiological key events leading to a toxic effect (Boobis et al., 2006; WHO, 2009; EFSA, 2008)

Adverse outcome pathway (AOP) is defined as the information on the causal links between a molecular initiating event (MIE), intermediate key events (Kes) and an adverse outcome (AO) of regulatory concern. 'a sequence of events from the exposure of an individual or population to a chemical substance through a final adverse (toxic) effect at the individual level (from a human health perspective) or population level (from an environmental perspective)' (Ankley et al., 2010; Meek et al., 2014; OECD, 2013). Such key events should be definable and make sense from a physiological and biochemical perspective and in a toxicity pathway. Early key events including the MIE have been defined by the OECD as the 'initial point of chemical-biological interaction within the organism that starts the pathway' (OECD, 2013).

### 3.1.3. About thresholds

The term "threshold" has a variety of different meanings, depending on the context in which this term is used.

As a matter of principle, the absence of an effect can never be proven experimentally and thus the existence of a "true" threshold in the mathematical sense remains controversial. According to Slob (1999), a dose-threshold may be defined in different ways:

- *Biological definition: The dose below which the organism does not suffer from any (adverse) effects from the compound considered.*
- *Experimental definition: The dose below which no effects are observed.*
- *Mathematical definition: The dose below which the response is zero, and above which it is non-zero.*

The World Health Organization (WHO, 2009) defines threshold as “Dose or exposure concentration of an agent below which a stated effect is not observed or expected to occur”. The WHO defines the threshold dose as “The dose at which an effect just begins to occur—that is, at a dose immediately below the threshold dose, the effect will not occur, and immediately above the threshold dose, the effect will occur. For a given chemical, there can be multiple threshold doses, in essence one for each definable effect. For a given effect, there may be different threshold doses in different individuals. Further, the same individual may vary from time to time as to his or her threshold dose for any effect. For certain chemicals and certain toxic effects, a threshold dose may not be demonstrable. The threshold dose will fall between the experimentally determined no-observed-(adverse-)effect level and the lowest-observed-(adverse-)effect level, both of which have been used by different scientific groups as a surrogate for the threshold dose in the performance of risk assessments”.

The WHO definition of a threshold dose indicates that there is no fixed value for a threshold. This applies both for the chemical as well as for the exposed individual. The discussion whether thresholds (experimental, mathematical, biological or “true”) exist or not and at what level (biochemical, individual, or population level) does not solve the problem that when the dose decreases, the dose-response curve becomes indistinguishable from the background response at a certain point and the shape of the dose-response curve remains unknown thereafter as this dose-range becomes experimentally inaccessible or non-observable. This point is largely dependent on the nature and the design of the study and its power to detect any effects. A biological threshold in this sense does not indicate a dose below which any response is zero, but a dose, below which the response may be considered to be biologically irrelevant provided sufficient power of the study.

Furthermore, thresholds may be discussed at different levels, e.g. at the molecular, cell, organ, individual, population or ecosystem level (Slob 1999). Thus, it is important to note that chemical risk assessment in the regulatory context usually addresses risks at the population level. Even if a particular threshold would exist at a certain level (e.g. for biochemical processes such as enzyme inhibition), it may no longer exist at higher levels of biological systems (e.g. at the organism or the population level) because the resulting dose response relationship is the result of a set of dose response curves.



#### 3.1.4. About critical effect

In toxicological risk assessment the critical effect is defined as the toxic or adverse effect occurring at the lowest dose of an agent. The critical effect level is the dose at which the critical effect starts to occur when increasing the dose. The critical effect level is linked to the size or magnitude of the effect, i.e. when it can be identified. The identification of a critical effect size is strictly related to a "normal" state and its natural background variability and when it becomes distinguishable from the background variation.

Ecotoxicological risk assessment is also often based on the toxic effect occurring at the lowest dose or concentration. A proxy for the critical effect level in environmental risk assessment is the use of the NOED or NOEC for these effects, but there are a number of exceptions (see Annex K).

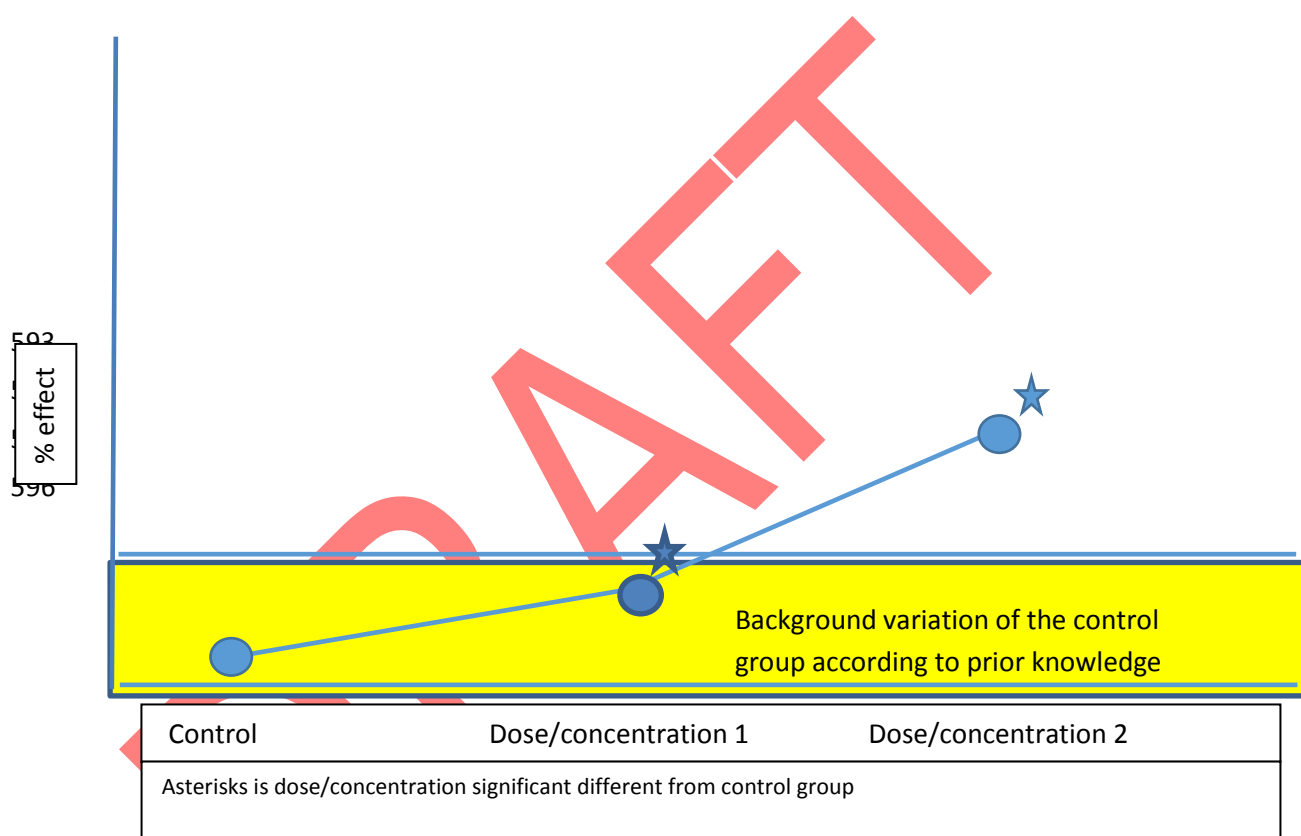
Assuming that temporal fluctuations in physiological parameters (e.g. haematology, biochemistry) in individual healthy non-exposed animals are non-adverse, the minimal magnitude of the Critical Effect Size (CES) fold change above background for a number of continuous parameters of toxicity studies can be derived (Buist HE, et al, 2009). If this "normal" range is exceeded this can be considered as a relevant effect size for this endpoint. The "normal" background range of a parameter may differ between individuals and between an individual and a population.

The size of an effect that would be considered biologically relevant should ideally be considered before answering the assessment question. (see also EFSA, 2017, chapter 2.5.2). Once this has been determined, a power analysis should be carried out to determine whether a study has sufficient power of detecting the defined effect as a statistically significant result, i.e. if the effect really exists. "Statistically significant" does not necessarily mean "important" or "meaningful" (or "biologically relevant"), as it is sometimes misinterpreted, but is a statistical statement on the property and information content of the observed data (EFSA, 2011). In other words, a statistically significant effect may exist, but may be biologically irrelevant because, although statistically significant, it is smaller than the predefined biologically relevant effect size, which can be defined based on its background variability. Conversely, lack of statistical significance should not be the sole rationale for concluding a lack of treatment- (exposure) related effect, just as statistical significance should not be the sole justification for concluding on the occurrence of a treatment-related effect (OECD, 2007).

An example of when a statistically significant treatment-related effect falls within the background variability for the control group according to prior knowledge and might be considered as irrelevant for risk assessment, is given in figure 2. The left point (value) in Figure 2 is the effect level observed in the control group. Note, that in this case the control value is at the low end of the background variability of the control group and although the middle point (value) is statistically significantly different from the control outcome, it is still within the background variation. In this particular case the value for the right point and not the mid point could be considered as the Lowest observed effect concentration or level (LOEC/LOEL).

Also in the guidance document on toxicity endpoints from avian and mammalian reproductive toxicity studies (EFSA, 2009b) it is mentioned that although the

magnitude of an endpoint in an exposed group could be statistically significantly different from that of the controls, it might not be biologically relevant. The following is a quote of this document: "In order to determine the biological relevance of an effect it should be considered whether the effect could lead to a functional deficit later on in the study, e.g. if a reduction in the weight of pups at birth leads to a decrease in level of survival. If not, then the effect may not be biologically relevant, however if there is a carry-over of effects into the number of survivors, it can be considered biologically relevant". That guidance document also provides more information for dealing with dose response relationships (see chapter 2.3.1 Determining toxicity endpoints from avian and mammalian reproductive toxicity studies (EFSA 2009b)).



**Figure 2: An example of when a statistical significant treatment-related effect falls within the background variability for the control group according to prior knowledge and might be considered as irrelevant for risk assessment**

### 3.1.5. About modelling approaches

In many risk assessments conducted by EFSA, modelling approaches related to biological relevance are used:

- To predict the value of endpoints, relevant to the assessment question, which cannot be measured at present time (e.g. spread of pathogens, see Annex A and Annex H);
- to estimate the value of biomarkers relevant to the assessed endpoints which cannot be measured directly, for instance as done in the example of setting dietary reference values in Vitamine D (Annex G);

- to estimate reference points for hazard characterisation in toxicological risk assessments by Benchmark Dose Modelling (EFSA, 2017)
- to assess large-scale or long-term effects on the biological system (Annex K);
- to extrapolate the outcomes of the risk assessment to various populations/receiving environments (Annex K);
- to assess the implications of uncertainties/assumptions on the outcomes of the risk assessment, for example sensitivity analysis;

Such models are quantitative or qualitative. As far as they are based on sound approaches and explicit assumptions, they can help risk assessors in understanding whether an effect size would be biologically relevant in various contexts (populations/ecosystems) and help risk managers make decisions.

### 3.1.6. About biomarkers

In studies on the interaction between an environmental agent and a biological system biological measurements are done. A wide range of such measurements are called biomarkers. The nature of these biomarkers is different and WHO identified three different classes of biomarkers: biomarker of exposure, biomarker of effect and biomarker of susceptibility.

For chemical agents a biomarker of exposure is defined as “an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism”. Urine, blood, faeces or nails are common media for the measurements of biomarkers of exposure.

A biomarker of effect is “a measurable biochemical, physiological, behavioural or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease”.

A biomarker of susceptibility is “an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance”. (WHO EHC 155, 1993.)

In relation to toxicity testing, it is important to note that a biomarker of effect provides information for an effect, but does not necessarily discriminate between adverse and non-adverse effects (Blaauboer et al, ALTEX, 2012). Its biological relevance depends on its relation to mode of action of an adverse effect or an adverse outcome pathway.

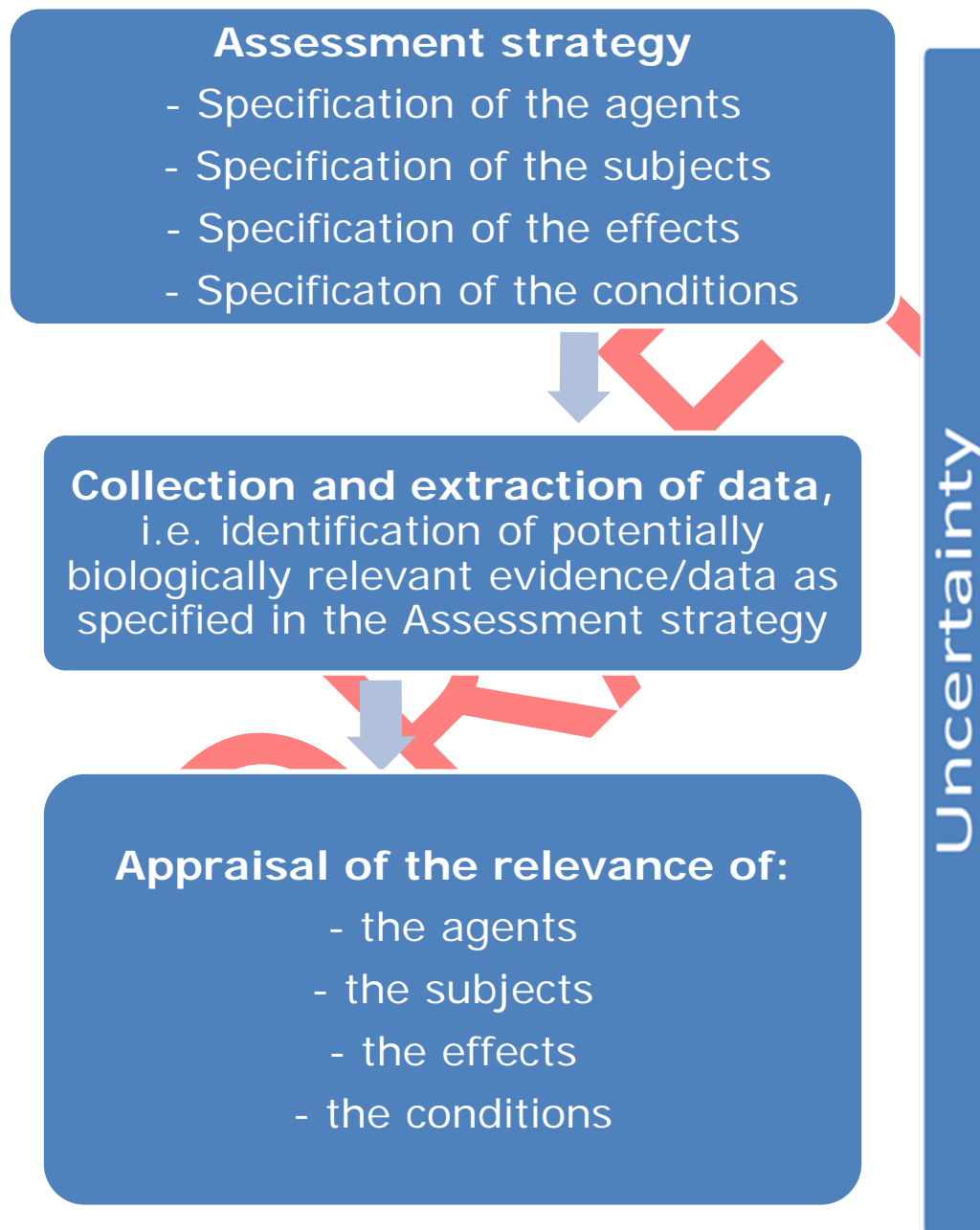
### 3.2. Framework for consideration of ‘relevance’

In the framework presented below (Fig.3) biological relevance is considered as described above, at three main stages related to the process of dealing with evidence:

- Development of the assessment strategy, in this context, specification of agents, effects, subjects and conditions.

- Collection and extraction of relevant data, i.e. identification of biologically relevant evidence/data as specified in the Assessment strategy
- Appraisal of the relevance of the agents, subjects, effects and conditions, i.e. reviewing dimensions of biological relevance for each data set.

In the course of the assessment, it might become apparent that additional data would be of relevance for the assessment and the process has to be reiterated.



**Figure 3: A framework of consideration of biological relevance at three main stages related to the process of dealing with evidence**

### 3.2.1. Development of the Assessment strategy and relation to biological relevance.

The assessment strategy should ensure that the assessment will answer the assessment question(s). When developing the assessment strategy the scope and objectives of the risk assessment should be carefully considered, and if necessary, clarified with the requestor. Further, if any questions arise in the course of the assessment regarding the objective and scope of the assessment, they require immediately to be addressed.

A number of relevance-related considerations should take place in the course of developing the assessment strategy. One of the main considerations is to identify and specify biological relevant data, before initiating the data collection. The following considerations depend on prior knowledge:

- Which is/are the **agent(s)** of interest for the assessment or activity assessed?
- What is/are the **subject(s)**, population(s) that should be covered by the assessment, are there any subgroups or sub-population particularly relevant that the assessment should address more specifically? Are there some specific levels of protection (e.g. 95-99% of the target population) to be considered? For environmental risk assessment, this issue is translated into the concept of protection goal (see glossary and EFSA 2016a)?
- What is/are the **effect(s)** associated with the exposure to the agent(s) that is/are considered as relevant for the assessment question?
- What are the relevant **condition(s)** regarding the exposure to the agent(s): route of exposure, exposure duration, timing of exposure etc.?

In standardised assessments, i.e. assessments that strictly follow guidelines or guidance documents, the assessment questions are generally already defined in a standard form, and there is also a standard procedure for assessing them. Sometimes also regulations prescribe what kinds of data are needed for the assessment. The standardised questions and procedures are part of what Codex (2015) refers to as 'risk assessment policy', defined as 'Documented guidelines on the choice of options and associated judgements for their application at appropriate decision points in the risk assessment such that the scientific integrity of the process is maintained'. This defines what questions are relevant to a class of assessments, and what effects, data and analysis are relevant for assessing them. It would also define what kind of biological data and effects that are relevant. According to the Codex, for standardised assessments, it is sufficient to confirm that the question defined by risk assessment policy is relevant to the case in hand. Where it is not, the assessor needs to interpret the

terms of reference and define relevant assessment questions in consultation with the decision-maker (Codex 2015)<sup>1</sup>, which in effect establishes the assessment strategy specific to the case in hand. The assessor then has to decide on what kind of biological data and effects would be relevant for the outcome of the assessment.

In many areas of EFSA's work where there are not standardised procedures, current practices for conducting common types of assessments have developed, for which an assessment strategy may be predetermined and documented, e.g. in guidance documents. This may imply that specific studies and data as well as specific outcomes are considered relevant. When using standardised procedures it is necessary to confirm that the procedure is relevant for the assessments at hand, but not necessary to reconsider the relevance of every element of the procedure. Hence, it is essential to recognise that all the considerations of specifying biological relevant data have to be done *de novo* for every assessment.

In cases where a standard procedure is not fully relevant for answering the questions asked by the requestor, the assessment becomes case-specific and the relevance of each element will need to be considered. This is consistent with the concept of standardised and case-specific assessments in the draft guidance on uncertainty (EFSA, 2016b).

### 3.2.2. Collection and selection of the biologically relevant data according to specifications

The relevance-related considerations described in the previous section on the development of the assessment strategy specify which evidence is relevant or irrelevant for the assessment and needed for answering the assessment questions with the minimum possible uncertainty. The assessment strategy should also serve as a basis for defining the protocol / strategy for data collection.

Following the application of the protocol / strategy for data collection, all the data and information collected should be evaluated for their relevance for the assessment.

Data of low quality should not be *a priori* considered irrelevant and excluded, as they may contain information important for the assessment. Instead, their implications should be considered, while taking into account the limited quality and associated uncertainty. The criteria for inclusion/exclusion of data should be explained and described within the risk assessment. The same applies for any established risk assessment guidelines, data quality criteria, default assumptions, decision criteria etc. that exist for the problem at hand. If data are excluded, this should be stated in the opinion along with the rationale for their exclusion.

<sup>1</sup> Codex (2015) states that the risk manager should establish risk assessment policy before risk assessment, in consultation with the risk assessor and other interested parties. In current practice for EFSA assessments initiative for this tends to lie with the assessor but, in principle, the decision-maker is responsible and should at least confirm their agreement.



It should be acknowledged that in the case of “standardised” assessments, the relevance of some evidence or data to be considered for the risk assessment is pre-set.

The legislation can also pre-determine the level of relevance of some of the evidence. This is the case for example for the validation of health claims where human data are considered as relevant to demonstrate and conclude on a positive effect, while other types of data (animal, in vitro or in silico) are only considered as supportive evidence. More on this will follow later in the document.

### 3.2.3. Appraisal of each data set collected

#### *Reviewing dimensions of biological relevance for each data set*

To review the relevance of a particular dataset, the assessor should go back to the relevance-related considerations to answer the assessment questions that have been identified during the problem formulation phase and development of the assessment strategy (see section 3.2.1):

##### *a) Relevance of the agent*

The assessor should consider whether the dataset or the study under consideration provides evidence directly on the agent subject to the assessment (e.g. nutrient, substance, microorganism, pathogen or invasive species). Studies providing indirect evidence on the agent of interest do have a certain amount of relevance to answer the assessment question; the fact that they do not address the agent of interest itself should be considered a relevance-related uncertainty and further characterised in term of impact on the assessment outcome (see section 3.2.4)

For instance when developing a farm-to-farm spread model in the case of the EFSA Scientific report “Schmallenberg virus: State of the art” (EFSA, 2014c) data on the related to Bluetongue virus were used for certain parameters in case data on Schmallenberg virus were lacking.

For chemical agents examples are the use of a structural analogue (QSARS or read-across), or a metabolite, or a precursor, or a pure compound for a formulation).

In a study by Cassard et al. (2014), mice models were used to evaluate the zoonotic potential of classical scrapie (See Annex C). A spectrum of strains is responsible for classical scrapie in sheep, and there may be variability in properties that affect the ability to cross the species barrier. In the study by Cassard et al. (2014), six different isolates of classical scrapie were used. The Biohazard Panel concluded that the isolates used in the study were relevant for the problem under investigation. However, evidence derived from a limited number of classical isolates cannot be extrapolated to represent the whole biological variability of classical scrapie.

##### *b) Relevance of the effect (nature and size)*

For each effect, the first step is to determine whether it is causally related to the exposure or treatment (for instance according to the Bradford-Hill criteria) (Hill, Austin Bradford.1965). Some considerations could be:

- Is the effect dose related?



- Is there potential confounding? Is the response a result of confounding?
- Does the exposure precede the response according to a plausible time scale?
- Is the effect biologically plausible; is there any information on the Mode of Action?

The objective of the next step is to determine whether the observed effect, in its nature and size, is relevant for the assessment question.

A wide range of assessment questions are considered by EFSA panels relating to many different agents such as nutrient, chemicals, microorganisms, pathogens, invasive species or inserted gene elements. Also the biological system assessed vary widely between EFSA Panels. Hence, a large variation in the effects caused by the agent can be expected. The scheme outlined below may be relevant to the assessment of chemical substances, but should also be applicable to other agents

Hence, as a first step considering biological relevance the assessor has to take into account the nature of the effect caused by the agent (e.g. nutrient, substance, microorganism, pathogen or invasive species) when addressing the assessment question. In this context the assessor may need to determine whether the effect in itself is adverse or beneficial and if not, whether it might be related to such an outcome. Size or magnitude of the effect may be important and is the other dimension to be considered when assessing the relevance of an effect.

A number of questions can help to decide on the (non-)relevance of the effect (see figure 4):

*1. Is the effect (in itself) an adverse or a positive effect (see section 3.1.1)?*

- Is the nature of the effect such that it is clearly adverse according to the WHO definition or beneficial (see 3.1.1). For continuous data, this may also be a quantitative question related to the size of the effect, which then have to be considered in a next step.
- Does the effect represent a homeostatic response? If so, is it within the homeostatic capacity of the organism or system? For continuous data, this may be a quantitative question related to the size of the effect (see also 3.1.1).
- Does the effect represent an adaptive response of a non-adverse nature? An example of such a response is the caecum enlargement, which is commonly seen in rodents as a result of a fibre rich diet. Another example is the stimulation of the immune system following exposure to microorganisms. One criterion to decide on a potential adverse effect is whether or not the effect seen occurs in isolation e.g. without pathological changes. (see also 3.1.1)?
- An example of a beneficial effect is supporting defence against pathogens in the upper respiratory tract, as measured by episodes of common cold and therefore biologically relevant (see Annex G). The

aim of the immune system is defence to pathogens, hence in case an agent helps to support defence to pathogens, in this case measured by reduction of the number of common cold episodes, the effect is considered beneficial. EFSA only accepts such effects when they are unequivocally demonstrated in the target species, i.e. the normal population, and only if exposure precedes the effect. If already existing common colds would be influenced by an agent in food, it would be considered a drug, which is outside of the remit of EFSA. Any effect of statistical significance in the proper direction would be considered beneficial. As pathogens are risk factors for infections, reduction of the load of such pathogens may also be considered as beneficial; the correlation of the load of pathogens and the infection they may cause needs to be known.

2. *If the effect (in itself) is adverse or positive, is the effect size of a sufficient magnitude to be considered relevant?*

In scientific assessments, the critical effect size of adverse or beneficial effects could be considered as the effect size that would be of sufficient magnitude to be biologically relevant. As discussed above (see 3.1.4) the critical effect level is directly linked to the critical effects size and can be defined as the concentration or dose in the concentration/dose response relationship at which an effect occurs or at which level the function of e.g. an organ, system or a (sub)population, will be changed. In all cases the normal or background variability of the endpoint should be taken into account (see chapter 3.1.4).

One way of taking into account natural variation of a biological system is equivalence testing. This can help to assess whether observed statistical differences are biologically relevant by comparing these observed differences with the natural variation of the biological system that is not exposed to the agent (i.e., responses to environmental or biological conditions other than the ones used in the assessment of the agent). While statistically significant differences may point at direct biological changes caused by the agent, they may not be relevant from the safety viewpoint. Equivalence testing may identify differences that are larger than normal natural variation and therefore help conclude on the biological relevance of the effects. Equivalence testing is currently being used in the safety assessment of GM plants and might also be used for other purposes (see Annex GMO example for further details) (Annex F).

A critical effect size can be determined by using expert judgement. This is seen in an example for lead where a benchmark response (BMR) of 1% was chosen based on the distribution of cognitive performance in the human population (EFSA, 2010). Another example is eggshell thinning and impact on egg cracking (Annex K) where the critical effect level, the biologically relevant percentage of egg shell thinning, starts at 18% when egg shell cracking begins to increase (EFSA, 2009b). In addition, models

can be used for setting a critical effect level. For example, models of focal species could be used to determine endpoints corresponding to cut-off values set by specific protection goals (SPG). These models can be used for calculating critical effect levels for certain types of effect, for instance for egg cracking, number of surviving chicks or the size of litters, above which the population of the focal species will be negatively affected to such an extent that the population will decline over time (see Annex K extended after public consultation to include modelling).

If it is not possible to determine a critical effect size for the adverse effect, the EFSA Scientific Committee recommends the use of default values. More specifically, a default critical effect size or benchmark response (BMR) of 10% (extra risk) should be used for quantal data and 5% (change in mean response) for continuous data from animal studies. As stated in the guidance, the default BMR may be modified based on statistical and biological considerations (e.g. when endpoint-specific information is available). The rationale for deviating from the default 5% BMR should be described and documented (EFSA, 2017).

For beneficial effects, the same principles apply as for adverse effects to decide whether the magnitude of the effect is biologically relevant. Very often, for beneficial effects, only statistical criteria are used, see for instance the example on health claims (Annex G). However, expert judgment using a weight of evidence approach should be applied to judge the relevance of the beneficial effects observed, i.e. to decide on the magnitude to consider an effect as relevant. Cut-off values should ideally be set a priori, but this is usually not done (see Annex F).

Another example is assessment of efficacy of feed additives with the capacity to increase the performance of chicken for fattening, providing positive economic effect for the farmer. Any such effect exceeding the costs of the additive can be considered as relevant. Hence, also the magnitude of the effect is of importance. In this case, however, the animal itself will not benefit from this positive effect. (see Annex E)

*If the effect is not in itself adverse or positive (e.g. a biochemical parameter), is it directly or indirectly linked to a(n) adverse/beneficial outcome?*

In determining whether an effect is linked to an adverse/ beneficial outcome, it should be considered, if the effect is a key event in the sequence of events leading to an adverse or beneficial outcome. In this context, one of the questions resulting from the risk assessment of BPA could serve as an example (see Annex D): 'What is the biological relevance for human health of the observed proliferative and morphological changes in the mammary gland following exposure to BPA and the possible relevance for the development of breast cancer'? Ductal hyperplasia and an increase of the number of terminal end buds may be regarded as supporting evidence for tumour formation along with an increase in the proliferation of epithelial cells. However, these proliferative changes do not need to be adverse by themselves, as epithelial cell proliferation is a normal physiological process in certain life stages and per se does not lead to tumour formation and even may be reversible.

However, it is generally accepted that under certain pathological conditions such as recurrent tissue damage and repair the proliferating tissue becomes more susceptible to tumour development.

Another well-known example is an alteration in circulating bioavailable thyroid hormone levels which may have a serious impact on organs or organ systems other than the thyroid itself, such as on the developing nervous system (see Annex I).

Some measured effects, such as liver enzyme induction, which may not be considered adverse in themselves, can have a modulatory influence on e.g. the toxicity of other agents.

An example of an indirect beneficial effect would be the addition of the enzyme glucanase to the feed of farm animals which has no significant nutritional value itself but which facilitates the intestinal digestion of cellulose, thereby enhancing the nutritional value of the feed. (EC, 1996)

*3. If the effect itself is directly or indirectly linked to a(n) adverse or beneficial outcome, is the effect size of a sufficient magnitude to be considered relevant?*

To assess this, similar considerations would apply as in point 2 above.

As an example, in the risk assessment of cadmium (see Annex J)  $\beta$ 2-microglobulin ( $\beta$ 2M) excretion in urine was used as a biomarker for kidney damage. Renal toxicity is characterised by cadmium accumulation in convoluted proximal tubules thereby causing cell dysfunction and damage, the earliest sign of which is the decreased absorption of low molecular weight proteins from primary urine and increased excretion of B2M. To determine the relevant size of the effect prior knowledge on the relationship between urinary excretion of  $\beta$ 2M and renal function or damage was used. A level of 300  $\mu$ g  $\beta$ 2M/g creatinine in urine was selected since exceeding this cut-off value has been associated with accelerated decline of renal function and increased mortality. As high level criterion a level above 1000  $\mu$ g  $\beta$ 2M/g creatinine was selected as exceeding this level would likely be associated with irreversible damage.

Another example is the use of biomarker to determine a population reference intake for vitamin D (EFSA, 2016d). The complexity of vitamin D metabolism and the unknown contribution of its endogenous synthesis do not allow determining a reliable vitamin D Average Requirement in the European population, hence calculating a Population Reference Intake for this population. The only possible approach relies upon the use of a biomarker (of status) - calcidiol or 25(OH)D - of which the serum concentration is related to bone health. Indeed, there is evidence of an increased risk of adverse musculoskeletal health outcomes below a certain threshold (50 nmol/L). Meta-regression analysis of the relationship between 25(OH)D serum concentration and total vitamin D intake allows to set an Adequate Intake of 15  $\mu$ g/d for the adult European population, an intake which should ensure that most of the adult population will achieve a serum 25(OH)D concentration near or above the target of 50 nmol/L. In this case, the relationships between 25(OH)D and adverse musculoskeletal health outcomes, on the one hand, and total vitamin D intake, on the other hand, were considered as the biologically relevant parameters.

In the re-evaluation of food additive aspartame the critical effects were identified as reproductive effects in several animal species including humans (Annex B). Phenylalanine concentration in plasma without damage to the offspring subtracted the level obtain from a meal was used as a cut-off value. A bolus dose of aspartame to a normal subject reaching this value was determined based on modelling. The current aspartame intake given the current ADI was well below the dose required in PKU heterozygous individuals and it was concluded that there was no safety concern. The figure below describes a general decision tree to decide whether a biological effect is relevant or not (Figure 4).

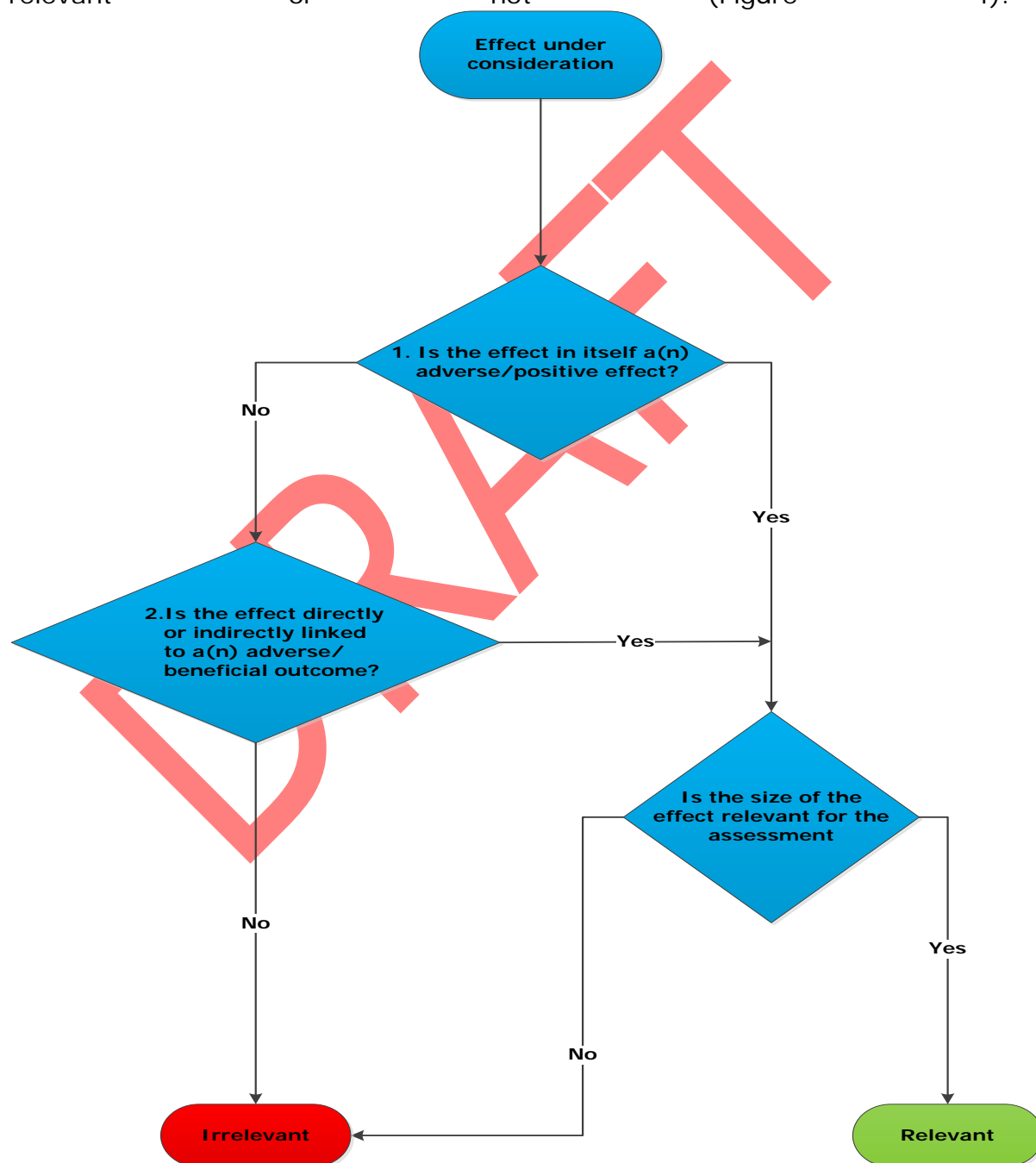


Figure 4: General decision tree to decide whether a biological effect is relevant or not



c) *Relevance of the subject*

In many cases proxies for the target species are used (e.g. rats instead of man, or standard organisms to represent a group of organisms) to test the biological effects. The conclusion on whether an effect is adverse/positive or not, is specific to the test system under investigation.

No direct extrapolation of the adverse or beneficial effects observed in experimental settings to humans/other species is generally possible. For example, if, following exposure to a chemical substance, a tumor occurs in one test species only or in an organ (e.g. Harderian glands; forestomach in rodents), which is not existing in humans, its relevance could be judged on the basis of the MOA. If the MOA is not known, additional information needs to be taken into consideration.

An example of a positive effect that depends on an organ only presents in certain species is utilisation of cobalt as precursor to vitamin B12 in rumen. The bacteria present in the rumen can metabolise inorganic cobalt into vitamin B12. Mammals without a rumen as in humans, are dependent on the uptake of exogenous vitamin B12.

In order to decide on the relevance of the test species to the human situation when testing chemicals, it is important also to understand the qualitative and quantitative interspecies differences, as well as the human variability in toxicokinetics (TK) and toxicodynamics (TD) processes. For a particular agent, the level of knowledge on TK and TD processes can range from very basic (external dose and toxicity) to a full quantitative understanding (external dose to internal dose to target organ dose and metabolism (TK) to specific target organ toxicity (TD) (EFSA, 2014d)

In farm animals, (Annex E) beneficial effects should be demonstrated in Efficacy Studies performed with the target animals. Extrapolations can be made for other categories of the target animals (e.g. from chicken for fattening to hens for laying, or from piglets to pigs for fattening) or other species (from dairy cows to other animals used for milk production, or from chicken to other avian species).

The relevance of information obtained from *in vitro* or *in silico* approaches needs to be considered in conjunction with knowledge on the MOA and other available information.

In case of biological hazards; species specific pathogenicity will be considered to decide whether the effect seen in the test species is relevant for the target species.

In a study by Cassard et al. (2014), animal models were used to evaluate the zoonotic potential of classical scrapie (See Annex C). Transgenic mice over-expressing the human PrP gene and homozygous and heterozygous for methionine and valine at codon 129 were inoculated intracerebrally. The Biohaz Panel concluded that the mouse lines were well established and have been shown to be susceptible to different CJD and BSE strains. Although over-expression of PrP is not a natural condition in humans, and it might have impact on some biological parameters, this can be considered a scientifically appropriate approach to modelling the molecular barrier for transmission of scrapie in humans despite some limitations of these transmission models.

1034 d) *Relevance of the conditions*

1035 The conditions of the test system should be looked at to decide on the degree of  
1036 the relevance to give to the resulting data in relation to the assessment question.  
1037 These include:

1038 Route of exposure

- 1039 • It is evident that the exposure as applied in the toxicity test should be as  
1040 close as possible to the exposure route expected in the field. This is not  
1041 always feasible. For instance, in environmental risk assessment for birds  
1042 and mammals the assessment of the acute risk is based on a gavage  
1043 study (LD50). This test does not really mimic normal exposure in the field  
1044 where bolus exposure rarely occurs. Animals exposed in nature are often  
1045 exposed via contaminated food over a period of time. In cases where the  
1046 exposure is the result of a more gradual exposure, the outcome may be  
1047 different compared with the bolus exposure (EFSA, 2005). The relevance  
1048 of the exposure conditions should be taken into account in the uncertainty  
1049 analysis..

1050 The route of exposure in a test system can sometimes be different from  
1051 that in the target system. An example of this is mice models that were  
1052 used in a study to examine the zoonotic potential of classical scrapie  
1053 (See Annex C). The mice were inoculated intracerebrally. However,  
1054 natural exposure to the classical scrapie agent in man is believed to  
1055 involve the oral route through the consumption of meat from an infected  
1056 animal. In this respect, the inoculation route used in the mouse model  
1057 does not represent an ideal strategy for the investigation of zoonotic  
1058 potential since the involvement of the digestive system, the rest of the  
1059 lymphoreticular system, the enteric nervous system and peripheral  
1060 nervous system have been bypassed by the direct deposition of the prions  
1061 in the brain. Therefore, it was concluded that the inoculation route used  
1062 by Cassard et al. (2014) cannot reproduce field conditions and does not  
1063 mimic natural exposure.  
1064

1065 Timing of exposure

- 1066 • For some compounds the timing of exposure is crucial. The test should  
1067 include the most sensitive period of the animal's life cycle. For instance,  
1068 some pesticides do hamper/prevent the moult of insects. When the  
1069 duration of the test does not include a moulting event of the tested  
1070 species the effect of the compound will not be shown.

1071 Duration of exposure

- 1072 • The duration of the test should mimic the duration of the exposure in the  
1073 field. In case the duration in the field is longer than the duration of the  
1074 toxicity test and the toxicity in the test did not reach a plateau (incipient  
1075 toxicity) it is possible that the outcome of the standard test does not  
1076 provide the answer that is needed for the risk assessment.

1077 Formulation or the vehicle used for exposure to the agent

- 1078 • By assessing the toxicity of the agent it should be assessed whether the  
1079 other compounds/additives added to the formulation do not influence the



outcome of the toxicity test (this can be both direction: a less toxic outcome or a more toxic outcome). It is also worthwhile to assess the vehicle used to apply the toxic compound to the test organism or the test system (often the vehicle is tested on its own).

#### Field studies (ref to the gd document for aquatic toxicity)

- For field studies additional criteria have to be checked, for instance, whether the important animal groups are represented in the field study. These types of criteria will not be discussed in this document, but are important issues to be considered when judging whether the outcome of a test can be used in risk assessment (see for instance the guidance document for aquatic organisms (EFSA 2013))

Other parameters, such as the number of animals per dose groups, number of doses tested, etc. are more related to reliability of the evidence (see the SC guidance on weight of evidence which is under development).

#### **3.2.4. Uncertainty related to the relevance**

Including evidence with less biological relevance adds to the overall uncertainty. Uncertainties arising when assessing biological relevance should be addressed and described together with other uncertainties at all stages of the assessment. General guidance on methods for assessing sources of uncertainty and their impact on assessment conclusions is provided by EFSA, 2016b, and can be applied to uncertainty arising from considering evidence that have limitations in their relevance as well as from other sources.

#### 4. Reporting the assessment of biological relevance

Assessing biological relevance should be addressed and described as part of the weight of evidence assessment. General guidance on methods for reporting weight of evidence assessment conclusions is provided by EFSA (under development), and can be applied to the assessment of biological relevance.

If the assessment of the biological relevance has been conducted following a standardised procedure previously established for use in this area of EFSA's work, the assessment of the biological relevance may be reported in the manner that is normal for that standardised procedure, provided this is transparent. The standardised procedure should be referenced and its applicability to the case in hand should be explained if it is not self-evident.

All other assessments of the biological relevance should be reported following the proposed framework according to the three basic steps of assessment of the biological relevance: (1) Development of the assessment strategy, including specification of agents, effects, subjects and conditions; (2) Collection and extraction of data, i.e. identification of potentially biologically relevant evidence/data as specified in the assessment strategy; (3) Appraisal of the relevance of the agents, subjects, effects and conditions, i.e. reviewing dimensions of biological relevance for each data set. This reporting should be included in the report of the weight of evidence assessment.

Reporting should be consistent with EFSA's general principles regarding transparency (EFSA 2006, 2009) and reporting (EFSA 2014a, 2015). The assessment of the biological relevance should include justifying the choice of methods used, documenting all steps of the procedure in sufficient detail for them to be repeated, and making clear where and how expert judgement has been used. Where the assessment used methods that are already described in other documents, it is sufficient to refer to those. Reporting should also include referencing and, if appropriate, listing or summarising all evidence considered, identifying any evidence that was excluded; detailed reporting of the conclusions; and sufficient information on intermediate results for readers to understand how the conclusions were reached.

Assessment of the biological relevance is part of the wider process of scientific assessment. Guidance on reporting other parts of the wider procedure, including evidence review, problem formulation and uncertainty analysis, is provided elsewhere (e.g. EFSA 2014b, EFSA 2015a, 2016b).

## 5. Conclusions and recommendations

- This guidance document is intended to guide EFSA panels and staff in the assessment of the biological relevance of scientific evidence. When addressing the mandate, the Scientific Committee acknowledged that the issue of biological relevance in risk assessment has a broader meaning than the biological relevance of an effect as described in the Terms of Reference. In fact, it encompasses also aspects related to the definition of the problem formulation. This, in turn, guides the development of the assessment strategy, which includes the decision on which data to use for the assessment (relevance of the data).
- Relevance is a fundamental concept in dealing with evidence and has different implications in terms of elements to be considered at different stages of the assessment and it can only be determined when the assessment question is well defined, which forms the basis for developing an assessment strategy.
- A framework was developed in which biological relevance is considered at three main stages related to the process of dealing with evidence:
  - Development of the assessment strategy, in this context, specification of agents, effects, subjects and conditions.
  - Collection and extraction of data, i.e. identification of potentially biologically relevant evidence/data as specified in the Assessment strategy
  - Appraisal of the relevance of the agents, subjects, effects and conditions, i.e. reviewing dimensions of biological relevance for each data set.
    - the agent; it should be considered whether the assessment is based on the agent of concern or on a surrogate agent.
    - the subject; in case proxies are used consider the relevance of effects occurring in these for the subject under assessment.
    - the effect; a wide variety of effects may be considered. Consideration should be given as to whether the effect is causally related to exposure to the agent, and the nature of the effect should also be taken into account, i.e. adaptive, directly or indirectly adverse or beneficial. Finally, it should be assessed whether the magnitude of the effect is sufficient to be of biological relevance and thereby of importance for the assessment outcome. It should be noted that the biological relevance of an effect can vary according to the assessment question.
    - the conditions; it should be considered whether the conditions of a test system, e.g. exposures, models, are relevant for the assessment question.

- Each step of relevance considerations may be source of uncertainty. The assessor should address these uncertainties as a part of the general uncertainty analysis of the assessment. The SC Guidance on Uncertainty (EFSA, 2016b) should be followed.
- The EFSA SC acknowledges that the diversity of fields covered by the different EFSA Panels impacts how the guidance could be implemented. More specific guidance for different areas might need to be developed.
- In implementing all the aforementioned recommendations, it is suggested that EFSA collaborate at the European and international level with relevant organisations and initiatives to harmonise developments in this area.

DRAFT

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## Examples of biological relevance considerations in panel-specific scientific assessments

### Annex A – AHAW

#### Case study of biological relevance in the area of animal health – Risk of introduction and establishment of Rift Valley Fever in the countries neighbouring the EU

##### Assessment strategy

Rift Valley Fever (RVF) virus is a vector borne virus that affects ruminants. Humans can contract the infection when in close contact with infected ruminants. The causative agent is a Bunyavirus that is endemically present in Sub-Saharan Africa. The European Commission wanted to know whether the virus is moving its territory in Northern direction.

Agent	Effect	Subject	Condition
Rift Valley Fever virus	Introduction into Mediterranean countries neighbouring the EU	Ruminant Population	Prevalence of the infection in Sub-Saharan Africa Movement of ruminants into region of concern Movement of vectors into region of concern
		Humans	

Agent	Effect	Subject	Condition
RVF virus	Establishment	Ruminant Population	Infection dynamics model
			Geographical density of ruminants
			Temperature in the geographical regions
			Geographical density of competent vectors (literature and further extrapolated based on suitability of the habitats)
			Temperature in the geographical regions

*Problem formulation:* What is the risk of entry and establishment of RVF into the Mediterranean countries neighbouring the EU.

##### Evidence/data needed to address the question

1371 Risk of Entry: Quantitative assessment of probability of introduction

- 1372 - Prevalence of the infection in source countries in Sub-Saharan Africa (data  
1373 originated from literature review and OIE outbreak information; in general data  
1374 availability was limited and available data was fragmented)
- 1375 - Data of movement of animals from source countries to region of concern (this is  
1376 undocumented trade, so no data available; estimates were made using formal  
1377 expert elicitation (Sheffield method))
- 1378 - Data on movements of competent vectors from source countries to region of  
1379 concern (data were derived from literature and expert elicitation)
- 1380

1381 Risk of Establishment: Modelling infection dynamics with regard to presence and density  
1382 of ruminants and competent vectors.

- 1383 - Infection dynamics model (a model described in literature was used)
- 1384 - Identifying competent vectors (these were derived from literature)
- 1385 - Geographical density of competent vectors (derived for a small part from  
1386 literature, further extrapolated based on suitability of the habitats)
- 1387 - Geographical density of ruminants (data derived from FAO)
- 1388 - Temperature in the geographical regions (based on temperature records of the  
1389 region)
- 1390

#### 1391 Data evaluation

##### 1392 Relevance of the agent

1393 Rift Valley Fever virus is a zoonotic virus of the family Bunyaviridae that has ruminant  
1394 species as reservoir hosts. Infected animals suffer from fever, young animals may die  
1395 and pregnant animals may abort. The infection is primarily transmitted between animals  
1396 through mosquitos. Humans can contract the infection when in close contact to animals.  
1397 Most infected humans follow a subclinical or mild (fever, headache, muscle pain) course,  
1398 but a small percentage of patients develops severe disease.

##### 1399 Relevance of the subject

1400 For the AHAW panel ruminants were the relevant subjects. These species are the  
1401 reservoir hosts of the virus. There is no evidence for sustainable human to human  
1402 transmission.

##### 1403 Relevance of the effect

1404 Two effects were examined: 1) Risk of introduction into the Mediterranean countries  
1405 neighbouring the EU and 2) Risk of establishment in those countries. Both are directly  
1406 related to the question asked by the requestor.

##### 1407 Relevance of the conditions

1408 For both the risk of introduction and the risk of establishment a mathematical model was  
1409 used to assess the risk. This is a generally accepted assessment, because it is not  
1410 possible to study these questions empirically. The introduction question included the  
1411 possible routes of infection, the contact rate, prevalence of the infection in the source  
1412 countries and likelihood of virus survival during the transport. Upon introduction the  
1413 infection may either fade out quickly after infecting only one or a few animals, or result in  
1414 extensive transmission, which is primarily dependent on the densities of ruminants and  
1415 that of competent vectors. Whether as a consequence of this spread the virus will  
1416 become endemic is dependent of the host population size (in a relatively small population

the influx of new susceptible animals might be insufficient for maintenance of infection) and climate (if temperatures drop in the winter to values that do not enable the vector cycle, infection will fade out in winter).

## Overall conclusion

The assessment revealed that the introduction of RVF in the region of concern is highly likely, but most often takes place in regions where the combined ruminant and vector density is insufficient to result in establishment. However, according to the assessment the region of concern has regions where RVF could become endemic.

## Uncertainty

The main potential sources of uncertainties in this setting may be summarised as follows:

- Mathematical models are a simplification of reality;
- Uncertainty around model parameters in particular :
  - Movement of animals from Sub Saharan to Northern Africa was derived using expert elicitation;
  - Vector density was mostly based on presence of suitable vector habitat only;
  - Uncertainty regarding vector competence of vectors present in Northern Africa
  -

The uncertainty around the number of introductions was high (but also the lower limit of the estimate indicates a likely introduction). Due to sparse data the uncertainty around the vector densities is also high and it is uncertain how the competence of vectors can vary within a certain vector species.

## Case study of biological relevance in the area of animal welfare – Gas stunning and unconsciousness at slaughter

### Assessment strategy

In the slaughter process animals are killed by exsanguination. However, in order to avoid pain and suffering, they should be rendered unconscious prior to exsanguination and remain so until death occurs through loss of blood. In most cases, poultry are stunned using an electric current, but recently gas stunning has gained interest due to animal welfare advantages. It is to be expected that the industry will continue to develop new stunning methods or modify electrical or gas stunning parameters. It is therefore important to ensure that the new or modified stunning methods meet animal welfare standards. Thus, an assessment protocol has been developed to evaluate new or modified stunning methods.

Agent	Effect	Subject	Condition
Stunning method	Loss of consciousness	Poultry	Slaughter

1450

*Problem formulation:* To maintain good standards of animal welfare, it is important to establish whether the new or modified stunning method (a) produces immediate loss of consciousness, (b) if loss of consciousness is not immediate, does it cause avoidable pain and suffering during the induction of unconsciousness, and (c) is the duration of unconsciousness long enough to avoid recovery of consciousness either prior to slaughter or during exsanguination.

## Evidence/data needed to address the question

Firstly, the brain mechanism associated with the induction of unconsciousness by a new or modified stunning method needs to be clearly explained. The state of consciousness can be ascertained under the controlled laboratory conditions by recording spontaneous as well as evoked activity in the brain using electroencephalograms (EEGs) before and after the application of a stunning method. The unique brain states that are incompatible with persistence of consciousness should be demonstrated using EEGs. Secondly, the correlation between EEG evidence and animal based indicators (as proxies) of unconsciousness for monitoring in slaughterhouses also need to be established. Thirdly, the duration of unconsciousness should be determined. In essence, the duration of unconsciousness should be longer than the sum time interval between the end of stunning and cutting blood vessels in the neck and the time it takes for the onset of death through exsanguination. Finally, the maximum permissible time between the end of stunning and neck cutting should be established.

#### **Data evaluation**

##### **Relevance of the agent**

Killing animals by exsanguination is a potentially painful process and the sources of pain includes, (a) cutting soft tissues, nerves and blood vessels in the neck (sawing motion or making several cuts), (b) direct activation of neurones by the blade as it transects the nerves produce intense pain and (c) the sensations produced during the injury discharge is likely to be an amalgam of all such inputs, and the overall effect is likely to be a sense of shock, comparable to an electric shock.

##### **Relevance of the subject**

Poultry is the relevant subject for the question, because it is also the target species for slaughter.

##### **Relevance of the effect**

The pain and suffering at exsanguination can be prevented by implementing pre-slaughter stunning of animals, i.e. rendering them unconscious prior to exsanguination.

##### **Relevance of the conditions**

The tests are done with the target species in a slaughterhouse setting. In this context, the brain of an animal is considered to be the seat of consciousness

#### **Overall conclusion**

Useful to test stunning methods according to the guideline

#### **Uncertainty**

Establishing neuronal correlates of unconsciousness remains to be a challenge. For example, the magnitude of changes occurring in the EEG considered to be incompatible with persistence of consciousness varies widely. The correlation between EEG criteria and animal based indicators of unconsciousness is not widely reported, and hence, rely on expert opinion.

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## Annex B – ANS

### Re-evaluation of aspartame (E951) as a food additive

#### Introduction

Following a request from the European Commission, the Panel on Food Additives and Nutrient Sources added to Food (ANS) of the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on the re-evaluation of aspartame (E 951) as a food additive.

Aspartame (E 951) is a dipeptide of L-phenylalanine methyl ester and L-aspartic acid bearing an amino group at the  $\alpha$ -position from the carbon of the peptide bond ( $\alpha$ -aspartame). The major hydrolysis and degradation products of aspartame are L-phenylalanine, aspartic acid, methanol and 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP).

For the purpose of the guidance and although the scientific assessment of Aspartame had a broader content, the example below focuses only on one effect.

Agent	Effects	Subjects	Conditions
Aspartame	Developmental effects	Rats and rabbits	Reproductive and developmental studies
L-phenylalanine Methanol Aspartic acid		Phenylketonuria (PKU) Patients	Humans heterozygous or homozygous for phenylalanine hydroxylase (PAH)

#### Assessment strategy

The re-evaluation of aspartame included the assessment of the safety of its gut hydrolysis metabolites methanol (which is oxidized to formaldehyde), phenylalanine and aspartic acid. The hepatic enzyme phenylalanine hydroxylase (PAH) is necessary to metabolize the amino acid phenylalanine to the amino acid tyrosine. When PAH activity is reduced, circulating phenylalanine levels will increase. Humans heterozygous for PAH mutations, show a slightly reduced capacity to metabolize phenylalanine compared to normal individuals. Individuals homozygous for PAH mutations, phenylketonuria (PKU) patients, have a markedly reduced capacity for phenylalanine metabolism. There is long established evidence for increased severity and frequency of adverse developmental effects with high phenylalanine plasma levels in human patients with phenylketonuria (PKU). Maternal PKU syndrome refers to the teratogenic effects of PKU during pregnancy. In untreated pregnancies wherein the mother has classic PKU with a plasma phenylalanine level greater than or equivalent to 1200  $\mu$ M (20 mg/dL), abnormalities in offspring occur at high frequencies.

The pathogenesis of this syndrome is unknown; it may be related to inhibition by phenylalanine of neutral amino acid transport across the placenta or to direct toxicity of phenylalanine and/or a phenylalanine metabolite (phenylpyruvic acid) in certain fetal organs.

1533 **Specification of the agent**

1534 Hydrolysis of aspartame in the gastrointestinal tract is essentially complete and there is  
1535 no systemic exposure to aspartame but systemic exposure to aspartic acid,  
1536 phenylalanine and methanol do occur.

1537 **Specification of the subject(s)**

1538 Adverse developmental effects have been reported in rats and rabbits treated with  
1539 aspartame as well as with phenylalanine. Due to the very efficient hydrolysis in the  
1540 gastrointestinal tract the amount of intact aspartame that enters the bloodstream has  
1541 been reported to be undetectable in several studies conducted in rats, dogs, monkeys  
1542 and humans.

1543 **Specification of the effect(s)**

1544 After birth, homozygous PKU babies show severe impairment in development and  
1545 cognition if the phenylalanine intake via the diet is not strictly controlled. Adverse  
1546 developmental effects were seen in children born to PKU patients and that these effects  
1547 appeared to be related to maternal phenylalanine levels. It has been reported that the  
1548 effects of phenylalanine in PKU mothers and their children both before and after birth had  
1549 developed considerably since the initial evaluation of aspartame.

1550 The MoA proposed for aspartame was that the toxicological effects observed in rats and  
1551 rabbits during pregnancy were due to the metabolite phenylalanine. It has been  
1552 postulated that phenylalanine could be responsible for some or all of the adverse effects  
1553 reported for aspartame in developmental toxicity studies with rats and rabbits.

1554 **Data Collection**

1555 The evaluation is based on original study reports and information submitted following  
1556 public calls for data, previous evaluations, and additional literature that became available  
1557 until the 15<sup>th</sup> November 2013.

1558 A complete package of embryotoxicity, reproductive and developmental toxicity studies  
1559 on aspartame in rats, mice and rabbits has been performed. Some of these studies were  
1560 also conducted with the aspartame metabolite phenylalanine.

1561 **Appraisal of the evidence**

1562 **Relevance of the agent(s) and the subject(s)**

1563 The results of the reproductive and developmental toxicity studies in rats indicated  
1564 NOAELs that ranged from 2000 to 4000 mg aspartame/kg bw/day. Developmental  
1565 changes in pup weight were observed at birth in studies at the dose of 4000 mg  
1566 aspartame/kg bw/day, which could be attributed to a combination of malnutrition and  
1567 nutritional imbalance due to excessive exposure to phenylalanine derived from  
1568 aspartame. This hypothesis was supported by the observation that administration of a  
1569 dose of phenylalanine equimolar to aspartame led to a similar decrease in maternal and  
1570 pup weight of rats, as observed in a concurrent aspartame group.

1571 The data from the reproductive and developmental toxicity studies performed with  
1572 rabbits were confounded both by the decrease in feed intake or the poor health of the  
1573 animals, and, in many cases by the number of deaths of pregnant rabbits in the treated  
1574 groups possibly related to misdosing during gavage treatment.

1575



**Relevance of the effect(s)**

The key effects observed in the reproductive studies with rats and rabbits related to a specific life stage acknowledged to be critical in both species and to humans. A spectrum of effects was observed in the rats and rabbits, particularly maternal toxicity and growth restriction of the offspring. The latter effect was recognized as an important outcome in humans because it was associated with an increased risk of perinatal mortality and morbidity.

**Relevance of the conditions**

The available reproductive and developmental toxicity studies on aspartame comprised nine studies, one in mice and eight in rats. In addition, eight embryotoxicity and teratogenicity studies were performed in rabbits, four with administration of aspartame by diet and four by gavage.

The best estimate of the critical effect level of phenylalanine exposure without damage to the offspring is 330 to 360  $\mu\text{M}$ . In calculating a safe level of aspartame exposure (based on plasma phenylalanine concentrations), the worst-case scenario was applied, that took into account that intake of aspartame occurs in combination with a meal leading to circulating plasma phenylalanine concentrations of 120  $\mu\text{M}$ .

The concentration of plasma phenylalanine derived from aspartame was, therefore, set to 240  $\mu\text{M}$  (i.e. 360  $\mu\text{M}$  minus 120  $\mu\text{M}$ ).

Based on **modelling**, a plasma phenylalanine concentration of 240  $\mu\text{M}$  would result from the administration of a bolus dose of 103 mg aspartame/kg bw to a normal subject.

For a PKU heterozygous individual the concentration of 240  $\mu\text{M}$  would be reached by the administration of a bolus dose of 59 mg aspartame/kg bw.

**Uncertainties**

The following main assumptions were made based on the proposed MoA:

- Phenylalanine plasma level of 360  $\mu\text{M}$  is the threshold for developmental effects.
- The diet results in phenylalanine plasma level not exceeding 120  $\mu\text{M}$ .
- Peak plasma phenylalanine concentration can be used in the dose-response modelling as surrogate of steady-state plasma phenylalanine concentration.
- Bolus administration of aspartame can be used in the dose-concentration modelling of plasma phenylalanine to represent a more typical pattern of aspartame intake.
- The 95th percentile confidence interval of the lower bound estimate of the aspartame dose- plasma phenylalanine concentration curve provides a safe limit for plasma phenylalanine for the entire population (with the exception of homozygous PKU patients).
- The increase in plasma phenylalanine concentrations following aspartame administration will be the same in the general population as in individuals heterozygous for PKU.
- Reproductive and developmental toxicity of aspartame is solely dependent on systemic exposure to phenylalanine.
- There is no requirement for a pharmacodynamic uncertainty factor (a sensitive human population (PKU patients) was used to define the threshold).
- There is no requirement for a pharmacokinetic uncertainty factor (the aspartame plasma phenylalanine concentration was based on a more sensitive human sub-population (PKU heterozygous)).

It was not possible to place a specific numerical value on the uncertainties related to these assumptions, but the aforementioned evaluations and considerations are more likely to overestimate than underestimate any potential developmental risk. Therefore it is not illogical to conclude that the results of the uncertainty analysis further support the conclusion, that there is no safety concern for aspartame at the current ADI in normal and heterozygous subjects.

## References

EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food), 2013. Scientific Opinion on the re-evaluation of aspartame (E 951) as a food additive. EFSA Journal 2013;11(12):3496, 263 pp. doi: 10.2903/j.efsa.2013.3496

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## **Annex C – BIOHAZ**

### **Case study of biological relevance in the area of biological hazards - Zoonotic potential of classical scrapie**

#### **Introduction**

Transmissible spongiform encephalopathies (TSEs) are a group of progressive conditions that affect the brain and nervous system of many animals, including humans. Unlike other kinds of infectious disease, the infectious agent in TSEs is believed to be a protein, called the prion protein. Misshapen prion proteins are transmissible and are able to induce abnormal folding of specific normal cellular proteins that are found most abundantly in the brain: they carry the disease between individuals and cause deterioration of the brain. TSEs are unique diseases in that their aetiology may be genetic, sporadic, or infectious via ingestion of infected materials and via iatrogenic means (e.g., blood transfusion). Prion diseases of humans include sporadic Creutzfeldt-Jacob disease (sCJD), new variant Creutzfeldt–Jakob disease (vCJD), Gerstmann-Straussler-Scheinker syndrome, fatal familial insomnia and kuru. Prion diseases of livestock include bovine spongiform encephalopathy (BSE) in cattle, classical scrapie in sheep and chronic wasting disease in cervids.

Host genetics substantially influence these diseases. In humans, familial prion diseases are closely associated with mutations in the prion protein gene, and the methionine/valine polymorphism at codon 129 appears to influence susceptibility, incubation period and in some respects disease phenotype.

One of the main questions in relation to the animal TSEs is their ability to infect humans. BSE is the only TSE agent identified as zoonotic. However, it has been hypothesised that other prions associated with animals such as classical scrapie can infect humans. For disease to develop in the case of exposure through foodstuffs, there must be exposure to a sufficient dose of the agent, the agent must be taken up from the gastrointestinal tract, enter the nervous system and be successfully transported to the neuronal cell bodies in the central nervous system. The infecting agent must then be able to 'convert' the cellular prion protein (PrP<sup>C</sup>) to the abnormal form of the prion protein (PrP<sup>Sc</sup>) at a rate which enables accumulation of sufficient PrP<sup>Sc</sup> to cause disease within the life-span of the host.

#### **Assessment strategy**

In a paper, 'Evidence for zoonotic potential of ovine scrapie prions', published in Nature, Cassard et al. (2014) studied the zoonotic potential of classical scrapie by bioassay in mice, in which a range of characteristics were assessed. These included incubation periods and neuropathological characteristics. The authors concluded that the results demonstrated that scrapie prions have zoonotic potential and raise new questions about the possible link between human and animal prions. The European Commission asked the BIOHAZ Panel to scientifically appraise the paper considering the limitations, assumptions and uncertainties associated with the study design and outputs.

In line with the framework set out in Figure 1 of the main document for consideration of relevance, the agent, effect, subject and conditions can be considered as follows:

Agents/Exposure	Effect/Outcome	Subject/Population	Conditions
Classical scrapie agent	TSE	Human	Oral exposure through the consumption of meat from an infected animal

1678

Hence, the problem can be formulated in these terms: in humans can the consumption of meat from classical scrapie infected sheep result in the development of a Transmissible Spongiform Encephalopathy?

#### 1682 Evidence/data needed to address the question

The evidence for the zoonotic potential of classical scrapie, as set out in the paper by Cassard et al. (2014), was evaluated by the Biohaz Panel using expert judgement.

#### 1685 Appraisal of the evidence

##### 1686 Relevance of the agent

A spectrum of strains is responsible for classical scrapie in sheep, and there may be variability in properties that affect the ability to cross the species barrier. There is experimental evidence that some isolates may not be completely stable, and their fundamental properties may shift on transmission. There is also potential heterogeneity of geographical distribution of individual strains.

In the study by Cassard et al. (2014), six different isolates of classical scrapie were used. These had been previously studied in other animal models and showed some degree of biological variability. The Biohaz Panel concluded that the deliberate selection of biologically variable scrapie isolates represents an important new aspect compared to previous studies on the subject, given the known diversity within the group of TSE agents identified as classical scrapie. The Panel further concluded that no case selection will conclusively and comprehensively ever represent the total potential field exposure, but the Cassard et al. (2014) study made a good, rational and supported choice of isolates designed to be distinct from one another, to represent some of the possible range of field strains.

In conclusion, the isolates used in the study by Cassard et al. (2014) were considered relevant for the problem under investigation. However, evidence derived from a limited number of classical isolates cannot be extrapolated to represent the whole biological variability of classical scrapie.

##### 1706 Relevance of the subject

In the study by Cassard et al. (2014), animal models were used to evaluate the zoonotic potential of classical scrapie. Transgenic mice over-expressing the human PrP gene and homozygous and heterozygous for methionine and valine at codon 129 were inoculated intracerebrally. Historically, laboratory studies using animal models were carried out using wild type mice. However, a substantial proportion of human and animal TSE isolates cannot be propagated into conventional mice models, which limits the usefulness of this system to characterize and compare TSE agents circulating in the field. More

recently, transgenic mice with PrP derived from different species have been increasingly used in experimental transmission of TSE agents. Transgenic mice that are homozygous for methionine at codon 129 of the human PrP gene have been previously shown to be fully susceptible to human TSEs, such as sCJD and vCJD and, to a much lesser extent, cattle BSE, the only animal TSE with confirmed zoonotic potential identified so far.

Different lines of transgenic mice that express the human PrP gene were used in the study, namely the tg340, tg361 and tg650 mouse lines. The tg340 mouse line expresses methionine approximately fourfold more than normal human brain tissue at codon 129. The tg650 mouse line overexpresses methionine sixfold at the same codon. The tg361 mouse line overexpresses valine at codon 129 at fourfold levels. A breeding cross between the tg340 and the tg361 provided mice that overexpress both M129 and V129 alleles at similar levels. Although PrP over-expression might circumvent the low susceptibility of gene-targeted tg mice, it is worth noting that an inevitable limitation of such transgenic mice is that only one human gene is present in the model, while disease susceptibility and incubation period are inevitably multi-factorial. Additionally, if the time taken for the conversion of human PrP<sup>C</sup> to PrP<sup>Sc</sup> exceeds the lifespan of the mouse, this may give a 'false negative' outcome.

The Biohaz Panel concluded that the mouse lines used by Cassard et al. (2014), in particular tg650 and tg340, are well established and have been shown to be susceptible to different CJD and BSE strains. Although over-expression of PrP is not a natural condition in humans, and it might have impact on some biological parameters, this can be considered a scientifically appropriate approach to modelling the molecular barrier for transmission of scrapie in humans despite some limitations of these transmission models, as mentioned above.

#### Relevance of the effect

In the study, serial passages were used in the transgenic mice for each of the six different strains of classical scrapie. Serial passages of bovine BSE, human sCJD isolates and human vCJD isolates were also carried out in these mouse lines for comparison with the classical scrapie isolates. Based on the attack rates observed after serial passages in transgenic mice, the potential for classical scrapie transmission is: i) low or absent in MM129 mice; ii) low or absent in MV129 mice; iii) absent in VV129 mice. The data suggest that BSE is still more efficient than scrapie in MM129 mice, while a single scrapie isolate would be more efficient than BSE in MV129 mice.

Moreover, the study also showed that the serial transmission of different scrapie isolates in humanised transgenic mice led to the propagation of prions that were phenotypically identical to those that cause sporadic Creutzfeldt Jakob Disease (sCJD) in humans.

In summary, the effect shown by Cassard et al. (2014) was considered relevant. The study showed that transgenic mice could be infected with classical scrapie strains but that the transmission was less efficient than for bovine BSE. It should also be noted that while serial passage maximises the chance of detecting the propagation of TSE agents, it does not mimic natural exposure in humans.

#### Relevance of the conditions

Intracerebral inoculation is a widely accepted and appropriate choice of inoculation in mouse models. This method can be used to assess the permeability of the transmission barrier at the molecular level, i.e. the conformational compatibility between the infecting

prion strain and the PrP<sup>C</sup> of the recipient species. This is an important factor which limits the propagation of prion agents among different species. Successful amplification of PrP<sup>Sc</sup> would indicate that the TSE strain has the potential to convert human PrP<sup>C</sup>.

Natural exposure in man is believed to involve the oral route through the consumption of meat from an infected animal. In this respect, the inoculation route used in the mouse model does not represent an ideal strategy for the investigation of zoonotic potential since the involvement of the digestive system, the rest of the lymphoreticular system, the enteric nervous system and peripheral nervous system have been bypassed by the direct deposition of the prions in the brain.

Therefore, it was concluded that the inoculation route used by Cassard et al. (2014) cannot reproduce field conditions and does not mimic natural exposure.

### Overall conclusion

The Biohaz Panel concluded that the paper by Cassard et al. (2014), provides evidence that some classical scrapie isolates can propagate in humanised transgenic mice and produce prions that on second passage are similar to those causing one form of sporadic Creutzfeldt-Jakob disease (sCJD). However, the Biohaz Panel also concluded that the results from the study raise the possibility that scrapie prions have the potential to be zoonotic but do not provide evidence that transmission can or does take place under field conditions.

### Uncertainty

The main potential sources of uncertainties in this experimental setting may be summarised as follows:

- Evidence derived from a limited number of classical isolates cannot be extrapolated to represent the whole biological variability of classical scrapie;
- The use of an animal model and the over-expression of PrP may not allow a direct extrapolation to human population;
- Subsequent serial passages were thought as a way to overcome the problem of allowing a longer incubation period in mice: the occurrence of a similar condition is not realistic in the field.
- The intracerebral inoculation route used by Cassard et al. (2014) cannot reproduce field conditions and does not mimic natural exposure.

### Reference

Cassard H, Torres JM, Lacroux C, Douet JY, Benestad SL, Lantier F, Lugan S, Lantier I, Costes P, Aron N, Reine F, Herzog L, Espinosa JC, Beringue V and Andreoletti O, 2014. Evidence for zoonotic potential of ovine scrapie prions. *Nature Communications*, 5, 5821.



**Annex D – CEF****Evaluation of the toxicity of BPA for humans considering all relevant toxicological information****Assessment strategy**

The EFSA scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) has re-assessed in 2015 the risks to public health from BPA exposure by evaluating the toxicity of BPA for humans, including for specific (vulnerable) groups of the population (e.g. pregnant women, infants and children, etc.) and considering all relevant toxicological information available. Exposure assessment was performed for various groups in the population and finally human health risks were characterised taking into account specific groups of the population [EFSA Journal 2015; 13(1): 3978].

Although the scientific assessment of BPA had a broader content, for the purpose of this guidance the example below focuses only on one effect.

**Specification of the agent**

Bisphenol A (BPA) is an organic chemical used as a monomer in the manufacture of polycarbonate plastics and epoxy resins and as an additive in plastics. Polycarbonates are used in food contact materials such as reusable beverage bottles, infant feeding bottles, tableware (plates and mugs) and storage containers. Epoxy resins are used in protective linings for food and beverage cans and vats.

The scientific debate on the risks for public health of BPA is focussed on its endocrine-active properties, which might adversely impact physical, neurological and behavioural development. In addition, other perturbations of physiology, both in animals and humans, have been brought in relationship to the endocrine-active properties of BPA. Among these are e.g. obesity, modification of insulin-dependent regulation of plasma glucose levels, perturbation of fertility, proliferative changes in the mammary gland possibly related to the development of breast cancer, immunotoxicity and adverse effects on the cardiovascular system (for an overview see NTP-CERHR, 2007, 2008, EFSA, 2006, 2008, 2010, and ANSES, 2011, 2013).

**Specification of the subject**

The question of interest resulting from this risk assessment of BPA was: 'What is the biological relevance for human health of the observed proliferative and morphological changes in the mammary gland following exposure to BPA and the possible relevance for the development of breast cancer'?

**Specification of the effects**

To update the risks to public health from BPA exposure, the complex BPA toxicity was re-evaluated by EFSA in 2015 using a Weight of Evidence (WoE) approach to identify the critical toxicological effects [EFSA Journal 2015; 13(1): 3978]. The effects on kidney weight were considered critical endpoints and taken forward to hazard characterization to assess a reference point (BMDL10) for the derivation of a health-based guidance value (TDI). As the scientific evidence for observed reproductive- and developmental-effects, neurological-, neurodevelopmental- and neuroendocrine-effects, immune effects, cardiovascular effects and metabolic effects was not sufficient, they were not taken

forward for risk characterisation, but these effects were included in the uncertainty evaluation.

In the WoE approach used for hazard identification, next to the general toxicity effects on kidney weight, also proliferative and morphological changes in the mammary gland were reported in several new toxicity studies and considered “likely” (likely refers to 66-100% probability), although no reference point (BMDL10) could be calculated.

These proliferative responses and possibly enhanced sensitivity to mammary gland carcinogens seen in animal studies might be of relevance for human health and were therefore included in the risk assessment.

#### Collection of data relevant to the problem formulation

Earlier evidence for BPA effects on cell proliferation and differentiation and morphological changes potentially related to tumour induction in the mammary gland [EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), 2010] were supported by new toxicity studies published from 2010 onwards. A number of these new laboratory animal studies in rodents (rats and mice, including a.o. transgenic mouse models and DMBA mammary tumour mouse model) and a monkey study, have reported effects on mammary tissue (mammary tumour induction, enhancement of mammary tumour growth and/or proliferative changes in mammary gland) after prenatal, perinatal and adult exposure to BPA.

Overall, using expert judgment the CEF Panel concluded that although there were methodological weaknesses in all these studies with the exception of a US FDA/NCTR subchronic toxicity study, which was a detailed guideline study conducted in accordance with GLP, they provide further evidence that BPA may enhance mammary epithelial proliferation in animal models.

However the proliferative changes in the mammary gland reported in these new studies, including a non-human primate study, are considered insufficient to conclude that there is a link to cancer development in later life. Nevertheless, there might be a possible role of BPA in increasing the susceptibility to mammary gland carcinogenesis.

#### Relevance of the agent

Although the exact mode of action in respect to the reported proliferative changes in the mammary gland is not clarified they may well fit with the conclusions of the mechanistic studies in which it is shown that BPA affects a number of receptor-dependent and independent signalling pathways, resulting in effects on hormone homeostasis and gene expression as well as cytogenetic and epigenetic effects.

It was concluded in the Panel that no single clearly defined mode of action of BPA can be identified that can contribute substantially to the understanding of the potential effects of BPA in humans. However, given that BPA appears to have multiple modes of action at the cellular level, and at least some of these MoAs involve cellular responses that are highly conserved across species (e.g. binding to oestrogen or androgen receptors), the relevance for humans of the variety of effects that have been reported for BPA in mechanistic studies cannot be totally discounted.

**Relevance of the subject**

Although there is no convincing evidence that BPA is carcinogenic in animals when exposed to adults or during pre- and post-natal (during lactation) development, a large number of the animal studies suggest that BPA can have a proliferative/developmental advancement effect on mammary tissue, and may also have an effect on tumour growth in animal models, particularly in sensitive transgenic models or when followed by a treatment with a complete carcinogen (DMBA).

In a large number of rodent toxicology studies, including a study with non-human primates, effects have been noted of pre- or perinatal BPA exposure on mammary gland morphology, cell proliferation and modification of gene expression. For instance the architectural modifications induced by the BPA in mammary glands of female offspring were transient increases in the total number of epithelial structures. Moreover, time- and dose-dependent modifications in gene expression profiles were observed after treatment with BPA, e.g. modulated (mainly up-regulated) genes related to cell proliferation, apoptosis and differentiation, cell communication, signal transduction, immunity, protein metabolism and modification. Supportive observations also came from several studies (10) using the subcutaneous route (s.c.) of administration of BPA, which also indicated that prenatal BPA exposure results in an increased cell proliferation/apoptosis ratio in normal tissue as well as preneoplastic lesions of rat mammary gland.

In relation to possible carcinogenic effects of BPA in animals when exposed pre- and post-natally (during lactation), several studies (5) used the dimethylbenzanthracene (DMBA) mammary tumour mouse model to assess the effects of fetal or postnatal exposure to BPA on the development of mammary tumour in adults. Overall, increased susceptibility to development of mammary cancer, decreased tumour latency and increased tumour multiplicity was reported.

Also studies were performed in transgenic mouse models, such as an adult knockout mouse model of mammary neoplasia, showing increased epithelial cell proliferation and hyperplasia in mammary glands of adult BRCA1\* knockout mouse upon BPA exposure via osmotic pumps. In addition, in a female transgenic MMTV-erbB2/neu mice susceptible to develop mammary carcinoma, BPA-treatment via drinking water resulted in a decreased tumour latency and increased tumour multiplicity, enhanced tumour volume and higher incidence of lung metastasis.

Also the US FDA/NCTR subchronic (90-day) toxicity study provided some evidence of a BPA-related effect in the mammary gland of female rats. Mammary gland duct hyperplasia of minimal severity was reported in the female groups examined at Post Natal Day (PND) 21 and in the high dose female BPA groups examined at PND 90.

Taken together, as intra-ductal hyperplasia in the mammary gland is observed in humans and is considered as a precursor of ductal carcinoma both in rodents and in humans, this lesion is considered of relevance when studied in animals (e.g. rodents) to predict cancer in the human mammary gland and is considered as adverse.

**Relevance of the effect**

Intra-ductal hyperplasia in the mammary gland is observed in humans and is considered as a precursor of ductal carcinoma both in rodents and in humans. Therefore, this lesion

1927 is of high relevance to predict cancer in the human and animal mammary gland and is  
1928 considered as adverse.

1929 Ductal hyperplasia and an increase of the number of Terminal End Buds (TEBs) may be  
1930 regarded as supporting evidence for tumour formation along with an increase in the  
1931 proliferation of epithelial cells. However, ductal hyperplasia may not always progress to  
1932 neoplastic lesions but may be reversible. Therefore, the relevance of these hyperplastic  
1933 lesions, in the absence of intra-ductal hyperplasia, is questionable for humans and the  
1934 level of adversity of these findings is unknown.

1935 Increased epithelial cell proliferation in the mammary gland of rodents is linked to  
1936 prolactin, which is also associated with an increased breast cancer risk in women. Thus,  
1937 an increase in prolactin levels constitutes an underlying mechanism in the induction of  
1938 cell proliferation, which may be indicative and therefore relevant for tumour promotion in  
1939 both the human and rodent mammary gland.

1940 In summary, based on the above indicated observations the proliferative and  
1941 morphological changes in the mammary gland reported in several toxicity studies with  
1942 BPA were considered relevant.

#### 1943 **Relevance of the conditions**

1944 The experimental test species and test conditions were for most studies considered  
1945 relevant, although the study reliability (e.g. data reporting, methodology) was considered  
1946 low or medium for all studies on BPA-induced proliferative effects.

1947 Although several studies were conducted with the non-relevant subcutaneous route of  
1948 pre-or perinatal BPA exposure, supportive observations of increased cell  
1949 proliferation/apoptosis ratio were reported in normal tissue as well as pre-neoplastic  
1950 lesions of rat mammary gland, while in other studies with perinatal BPA exposure no  
1951 such lesions were detected.

1952 The relevance of the findings in the DMBA mammary tumour model and the sensitive  
1953 transgenic models is uncertain because of limited experience with these models.

#### 1954 **Uncertainties**

1955 The uncertainties related to the induction of proliferative changes in the mammary gland  
1956 following BPA administration, i.e. intraductal hyperplasia, epithelial cell proliferation and  
1957 ductal hyperplasia (including increase in the number of TEBs), were evaluated taking into  
1958 account the reliability of the study results.

1959 For the evaluation of uncertainty the expert panel reviewed the studies considered in the  
1960 WoE, and extracted key information from each study and collated that in a graphical  
1961 format. The graphs summarised for each study, the life stage of the animals at treatment  
1962 onset, duration of treatment and sampling time for measurements, the doses tested,  
1963 whether there was a statistically significant effect at any dose, the number of strengths  
1964 and weaknesses of the study, the Panel's evaluation of the reliability of the study and its  
1965 relevance to the effect of interest.

#### 1966 **Some potential sources of uncertainties:**

1967 - Several studies with subcutaneous, pre- or perinatal BPA exposure reported on  
1968 intraductal hyperplasia in the mammary gland (i.e. an increase in the relative number of  
1969 ducts lined by three or more layers of epithelial cells), while in other studies with  
1970 perinatal BPA exposure no such lesions were detected.

1971 - Whilst epithelial cell proliferation is a normal physiological process in certain life stages  
1972 (pre-/perinatal period, pregnancy) and per se does not lead to tumour formation, it is  
1973 generally accepted that under certain pathological conditions such as recurrent tissue  
1974 damage and repair the proliferating tissue becomes more susceptible to tumour  
1975 development. In studies with rats treated with BPA and, thereafter, with a well known  
1976 complete carcinogen (DMBA), as well as in studies with transgenic mice, increased cell  
1977 proliferation was reported along with tumour formation. In case of the study that  
1978 observed cell proliferation in transgenic mice which spontaneously develop tumours, the  
1979 relevance of these findings to whether proliferative changes occur at low BPA doses in  
1980 normal animals was considered medium, taking into account the increased sensitivity of  
1981 this mouse model to tumour development.

1982 - Increase in the number of terminal end buds (TEBs) as well as ductal hyperplasia was  
1983 reported in several studies even at very low BPA doses. However, it should be noted that  
1984 these putative pre-neoplastic lesions may be reversible and will not in all cases progress  
1985 to neoplasia.

1986 - In addition also the study reliability (e.g. data reporting, methodology) was considered  
1987 low or medium for all studies on BPA-induced proliferative effects.

## 1988 Conclusion

1989 In the final assessment the overall likelihood of the BPA-induced proliferative changes in  
1990 mammary gland in animals exposed during pre- and postnatal (during lactation)  
1991 development or up to 90 days (gavage) was considered "likely", and taken forward for  
1992 the risk characterisation based on the consistency of the effect in a number of studies.

1993 The Panel concluded that the health-based guidance value should cover the lowest dose  
1994 in the dose range for which the likelihood approaches "likely" from the overall uncertainty  
1995 evaluation, taking into account uncertainty of all the evaluated endpoints as well as their  
1996 relevance and adversity to humans. The uncertainty evaluation approached "likely" in the  
1997 (HED) dose range of 100-1000 µg/kg bw per day. The Panel therefore decided that the  
1998 uncertainty regarding the above mentioned effects at the HED of 100 µg/kg bw per day  
1999 and higher should be taken into account when establishing a health-based guidance  
2000 value by including an extra factor in establishing the TDI. Thus, as the reference point  
2001 was 609 µg/kg bw per day based on the mean relative kidney weight and the lower end  
2002 of the dose-range for which the uncertainty evaluation for other endpoints approached  
2003 "likely" is 100 µg/kg bw per day, a factor of 6 was applied.

2004 The CEF Panel applied finally a total uncertainty factor of 150 for inter- and intra-species  
2005 differences (1 for toxicokinetics and 2.5 for toxicodynamics and 10 for intra-species  
2006 differences), and the uncertainty factor of 6 (for e.g. mammary gland effects) to  
2007 establish a temporary Tolerable Daily Intake (t-TDI) of 4 µg/kg bw/d.

2008 By comparing the t-TDI with the exposure estimates, the CEF Panel concluded that there  
2009 is no health concern for any age group from dietary exposure or from aggregated  
2010 exposure.

## 2011 References

2012 EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and  
2013 Processing Aids), 2015. Scientific Opinion on the risks to public health related to the  
2014 presence of bisphenol A (BPA) in foodstuffs: Executive summary. EFSA Journal 2015;13  
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## Annex E – FEED

### Example from FEEDAP

#### 1. Problem formulation

The scientific evaluation of feed additives by EFSA includes:

- The safety of the additive for the target animals
- The safety of the additive for the consumer (human health)
- The safety of the additive for the user/worker
- The safety of the additive for the environment
- The efficacy of the additive

The assessment of feed additives is a standardised process, which follows legal guidelines and guidance documents. The assessment questions are already defined in a standard form in the terms of reference and the assessment follows a standardised procedure.

The evaluation of the biological relevance of an effect under consideration in the FEEDAP Panel includes adverse (unwanted) effects and positive (wanted) effects on potentially different species and has to address all aspects indicated above.

The feed additive considered in this example consists of two essential oils derived from steam distillation of *Thymus vulgaris* (thyme) and *Illicium verum* (star anise), quillaja bark powder, crushed herbs and spices, and other feed materials (EFSA FEEDAP Panel, 2016). Thymol and trans-anethole are the major components of thyme oil and star anise oil, respectively. Star anise oil may contain the alkenyl-benzene derivative estragole in considerable concentrations.

The additive is intended for use in chickens for fattening (the target species) at a dose of 150 mg/kg complete feed.

Problem formulation at a glance is shown in the following Table.

Agents	Effects	Subjects	Conditions
Additive/Single active substances: <i>trans</i> -anethole and thymol	Short-term toxicity (mortality, clinical effects, performance parameters, haematology, blood chemistry, histopathology when needed)	Target species	Dietary exposure at recommended dose in feed (150 mg/kg feed) – Establishment of a safe dose via tolerance studies (x10 overdosing)
Single active substance: <i>trans</i> -anethole	Liver effects for <i>trans</i> -anethole in laboratory animals	Target species, Consumers	Dietary exposure via residues (data available from tolerance study, x10)
Single active substances: <i>trans</i> -anethole and thymol	Irritation, sensitisation skin	Users/workers	Exposure by inhalation, contact, systemic exposure
Single active substances: <i>trans</i> -anethole and thymol	Short-term effects (LC50, EC50) and long-term effects (NOEC)	Terrestrial and aquatic organisms in the environment	Exposure via manure containing residues or non consumed feed

Agents	Effects	Subjects	Conditions
Single active substance: estragole	Genotoxicity	All subjects	See above
Additive/Single active substances: <i>trans</i> -anethole and thymol	Improving animal performances	Target species	Dietary exposure at the proposed use level
	Digestibility enhancer		

2070

## 2071 Specification of the agents

2072 The active substances in the additive were considered to derive mainly from the thyme  
 2073 oil and star anise oil. The crushed herbs and spices will also contribute to the activity but  
 2074 to a lesser extent. Thymol and *trans*-anethole, the major components of thyme oil and  
 2075 star anise oil, represent about 0.2-0.4% and 4-5% of the additive, respectively. Star  
 2076 anise oil may contain the alkenyl-benzene derivative estragole up to 6% (European  
 2077 Pharmacopoeia, 2005).

2078 Hepatotoxic effects have been reported for *trans*-anethole. This compound is therefore a  
 2079 relevant compound to consider.

2080 Estragole was demonstrated to be genotoxic in several *in vitro* and *in vivo* assays using  
 2081 mammalian cell systems and carcinogenic in mice after oral administration. Thus,  
 2082 estragole is a critical agent in the risk assessment process.

## 2083 Specification of the effect(s)

### 2084 Adverse effects

2085 Liver effects: Hepatotoxic effects have been reported for *trans*-anethole when  
 2086 administered to rats (WHO, 2000, EFSA FEEDAP Panel, 2011a). A no observed effect  
 2087 level (NOEL) of 300 mg/kg bw per day was derived from a 90-day study based on  
 2088 elevated serum activity of  $\gamma$ -glutamyl transferase observed at 600 and 900 mg/kg bw per  
 2089 day in male and female rats. The NOEL was considered as an appropriate point-of  
 2090 departure to derive an acceptable daily intake (ADI) of 0-2.0 mg/kg bw per day (by  
 2091 applying an uncertainty factor of 200 to allow for deficiency in the long-term study;  
 2092 WHO, 2000).

2093 Genotoxicity: Estragole was demonstrated to be genotoxic in several *in vitro* and *in vivo*  
 2094 short term assays and carcinogenic in mice after oral administration. As such, estragole  
 2095 has the ability to induce cancer in the exposed organisms through a genotoxic mode of  
 2096 action (MoA); as a genotoxic agent, it is considered also to induce mutations in germ  
 2097 cells of humans and animals, with negative effects for the reproduction. Both effects are  
 2098 clearly defined as adverse and could be relevant for target animals, consumer, user and  
 2099 environment, if the conditions of the exposure to the compound allow the adverse  
 2100 outcome to occur.

## 2101 Positive effects

2102 An assessment of the efficacy of the feed additive is needed because the applicant claims  
 2103 that it increases the animal performance and digestibility of feed in chickens for  
 2104 fattening. Relevant performance parameters suitable for the assessment are the  
 2105 determination of feed intake, body weight gain and feed to gain ratio. Trials to  
 2106 demonstrate the efficacy of feed additives *in vivo* should be performed according to the  
 2107 guidance published by EFSA (EFSA FEEDAP Panel, 2011b).

**2108 Specification of the subjects****2109 Adverse effects**

2110 The subjects of adverse effects are: (i) the target animal fed diets containing the additive  
2111 (chickens for fattening), (ii) the consumer of the food products from chickens fed the  
2112 additive, (iii) the workers handling the additive and (iv) terrestrial and aquatic organisms  
2113 in the environment.

**2114 Positive effects**

2115 The subjects of positive effects are the target animals fed diets containing the additive  
2116 (chickens for fattening).

**2117 Specification of the conditions**

2118 trans-Anethole and estragole are part of a diet for chickens. Thus, the compounds enter  
2119 the body of the animals by oral uptake. Possible residues of the additive/active  
2120 substances in the meat from chickens fed the additive are taken up by humans with their  
2121 food. Users and workers, handling the additive or the feed with the additive may also be  
2122 exposed to the compound via the skin, eye, mucosae or lung. Organisms of the  
2123 environment may be exposed to the compounds or their metabolites via the manure of  
2124 the chicken, which is used as a fertilizer and could contain residues of the additive.

**2125 Collection of data relevant for the problem formulation**

2126 The assessment is based on evidence/data provided by the applicant in the form of a  
2127 technical dossier, prepared following the provisions of Regulation (EC) No 429/2008 and  
2128 relevant Guidance documents (EFSA, 2008; EFSA FEEDAP Panel, 2011b, 2012a,b,c).

2129 These data included the characterisation of the additive, two tolerance studies in  
2130 chickens for fattening, residue data in meat and liver from chickens fed the additive at  
2131 10x the recommended dose, five long-term and six short-term efficacy studies.

2132 Tolerance studies are designed as short-term toxicity studies to assess adverse effects of  
2133 the additive in the target species at the proposed conditions of use and at x-fold (10x,  
2134 100x) the recommended dose. The endpoints considered in tolerance studies are:  
2135 mortality, clinical effects, zootechnical parameters (body weight, average daily gain,  
2136 average daily feed intake, feed conversion ratio), haematological and blood chemistry  
2137 parameters, gross pathology, organ weight and histopathology (if needed).

2138 Efficacy studies are designed to demonstrate the efficacy of the additive at the lowest  
2139 recommended dose. A significant effect on the performance parameters consistently  
2140 observed in three long-term studies (feed intake, body weight gain and feed to gain  
2141 ratio) allows the conclusion that the additive has the potential to be efficacious.

**2142 Assessment of the collected data sets for biological relevance****2143 trans-Anethole****2144 Relevance of the agents****2145 Adverse effects**

2146 trans-Anethole specified as a major component of the additive is considered, at least  
2147 in part, responsible for potential adverse effects of the additive. Literature data are  
2148 available for the adverse effects of the pure compound trans-anethole (liver toxicity in  
2149 rat, WHO, ).

2150 If adverse effects were observed in tolerance studies performed with the additive, it  
2151 would not be possible to conclude which agent(s) is (are) considered responsible for the  
2152 observed effects. Besides trans-anethole, other additive ingredients or components of the  
2153 essential oils could also be responsible for potential adverse effects of the additive.

2154

## 2155 Positive effects

2156 The efficacy of the feed additive containing thymol and trans-anethole as part of  
2157 essential oils and thus its biological relevance was demonstrated by a statistically  
2158 significant increase ( $p < 0.05$ ) of the performance parameters indicated above, however,  
2159 there is no direct evidence that this effect is due only to these two components.

## 2160 Relevance of adverse effects of trans-anethole

### 2161 Relevance of the methods

2162 Possible adverse effects for target species were assessed in tolerance studies, where the  
2163 additive was fed at the proposed conditions of use and at 10-fold the recommended  
2164 dose. These tolerance studies included endpoints which could also detect adverse effects  
2165 on the liver (liver weight, liver enzymes, etc.), which were observed in the rat studies.  
2166 Since no adverse effects were observed at the proposed conditions of use and at up to a  
2167 10-fold of the recommended dose, it was concluded that the additive and the active  
2168 substances are well tolerated by the target animals.

### 2169 Relevance for the target species

2170 Liver toxicity was observed in sub-chronic and chronic toxicity studies in rats treated with  
2171 trans-anethole. These effects are not specific for rats and can be extrapolated to other  
2172 species. They are therefore considered relevant for the target species, i.e. chicken for  
2173 fattening. However, the conditions of chronic studies could be of limited relevance for  
2174 target species with a short life span as is the case for the target animals of this example.

2175 Tolerance studies performed with the additive under assessment are relevant to assess  
2176 adverse effects in the target species. Liver effects were not observed in tolerance studies  
2177 in chicken for fattening. The reason might be, that the exposure level of trans-anethole  
2178 as part of the feed additive was not high enough. Assuming that the additive is supplied  
2179 at the proposed use level of 150 mg/kg and considering the default values of feed intake  
2180 and body weight for chickens for fattening (EFSA FEEDAP Panel, 2012d), it can be  
2181 calculated that this dose level would result in an exposure of 0.3 mg/kg bw of trans-  
2182 anethole per day. In the experiment with the 10-fold overdose of the additive this value  
2183 would be 3 mg/kg bw per day. The NOEL derived from the 90-day rat study was 300  
2184 mg/kg bw, which provides a 1000-fold margin of safety compared to the chicken exposed  
2185 with the proposed dose level of the additive (150 mg/kg feed) and a 100-fold margin for  
2186 the experiment with the 10-fold overdose. Thus, the liver toxicity of trans-anethole is not  
2187 relevant for the target animals because the exposure level is not high enough.

### 2188 Relevance for the consumer

2189 trans-Anethole is metabolised along the same three major pathways in rat, mice and  
2190 humans. Therefore, hepatotoxicity in rats was considered relevant to humans, if they are  
2191 exposed to trans-anethole via residues.

2192 The applicant provided evidence that residues of trans-anethole could not be detected in  
2193 meat from chickens fed the additive at 10 times the recommended dose (limit of  
2194 detection, 0.1 µg/g). Exposure of consumers to trans-anethole can therefore be  
2195 excluded. The presence of trans-anethole in chickens feed at the recommended dose  
2196 level will therefore not be of biological relevance for the consumer.

### 2197 Relevance for the user

2198 trans-Anethole is irritating to skin and may cause risk of serious damage to eyes after  
2199 direct exposure. Handling of the compound during preparation of the additive could  
2200 therefore provide adverse effects to workers. Beside trans-anethole, the feed additive  
2201 contains a variety of other compounds, which have the potential to irritate eyes and  
2202 mucous membranes and to cause allergies upon contact with skin and respiratory  
2203 organs.

2204

**2205 Relevance for the environment**

2206 trans-Anethole present in the feed of chicken for fattening will be extensively  
2207 metabolised to inert compounds, excluding possible biologically relevant effects on the  
2208 environment.

**2209 Relevance of positive effects for the target animals**

2210 The applicant claims that the feed additive increases the performance of chicken for  
2211 fattening. This effect was proven in experimental trials with the target animals. Statistical  
2212 parameters are used to confirm this claim. The effect cannot be attributed to certain  
2213 compounds of the complex composition of the feed additive. The effect is of economical  
2214 relevance for the farmer, because it reduces the costs for the meat production.

**2215 Estragole****2216 Relevance of the agent**

2217 A battery of standardized test systems is available to prove the genotoxicity and  
2218 carcinogenicity of chemicals. The EFSA guidance on genotoxicity testing strategies  
2219 applicable to food and feed safety recommends (EFSA SC, 2011) "a step-wise approach  
2220 for the generation and evaluation of data on genotoxic potential, beginning with a basic  
2221 battery of in vitro tests, comprising a bacterial reverse mutation assay and an in vitro  
2222 micronucleus assay. (...) In case of positive in vitro results, review of the available  
2223 relevant data on the test substance and, where necessary, an appropriate in vivo study  
2224 to assess whether the genotoxic potential observed in vitro is expressed in vivo is  
2225 recommended. Suitable in vivo tests are the mammalian erythrocyte micronucleus test,  
2226 transgenic rodent assay, and Comet assay. If the in vivo assay results in positive effects,  
2227 the substance should be considered as an in vivo genotoxic agent. (...) If a two year  
2228 carcinogenicity study in rodents results in a significant increase in the formation of  
2229 malignant tumours compared to the control, the compound is considered as an animal  
2230 carcinogen and possible human carcinogen."

2231 Evidence exists for the genotoxicity of estragole in V79 cells, CHO cells as well as rat and  
2232 human hepatocytes in vitro and ex vivo, after oral treatment of rats with estragole  
2233 (Martins et al., 2012). Estragole was also clearly genotoxic in transgenic mouse and rat  
2234 strains (Suzuki et al., 2012). Clear evidence for the carcinogenicity of estragole comes  
2235 from studies in mice (Drinkwater et al., 1976).

2236 The MoA for the genotoxicity of estragole is the oxidation to 1-hydroxyestragole by  
2237 CYP1A2 and conjugation with sulfate to 1-sulfoxyestragole by SULT1A1 (Wiseman et al.,  
2238 1985). Spontaneous abstraction of SO<sub>4</sub><sup>2-</sup> releases a carbocation, which forms adducts  
2239 with DNA and proteins. The formation of such adducts was demonstrated in the liver of  
2240 mice and other mammalian species including human liver specimens in vitro (Phillips et  
2241 al., 1984; EMA, 2014). On the basis of this mechanism, estragole is a genotoxic  
2242 hepatocarcinogen and the formation of DNA adducts is the first pre-initiation step.  
2243 Although hepatocarcinogenicity of estragole has only been demonstrated in mice, the  
2244 presence of the enzymes involved in the critical steps of tumour initiation is not restricted  
2245 to mice and makes it likely that the same MoA takes place in other species including  
2246 birds and humans (EMA, 2014). In the absence of evidence showing that estragole does  
2247 not reach germ cells, it has to be assumed that estragole can exert its genotoxic effects  
2248 in both somatic and germ cells.

**2249 Relevance of adverse effects of estragole**

2250 Although there is a debate about the question whether or not a threshold dose exists for  
2251 genotoxic compounds, it is generally accepted that the exposure of humans and animals  
2252 to carcinogenic compounds should be avoided as much as possible. With respect to the  
2253 food and feed industry this means that, whenever possible, carcinogens should not be  
2254 added to human food or animal feed. Therefore, the presence of the genotoxic and  
2255 carcinogenic potential of estragole in the feed of farm animals, which serve as food for  
2256 humans is of critical relevance for the risk assessment.



**2257 Relevance of the subjects and conditions****2258 Relevance for the target animal**

2259 The additive is intended as a feed additive for chicken for fattening. These animals have  
2260 a short life span, which makes it very unlikely that they develop cancer as a result of the  
2261 exposure to the carcinogenic compound in their diet. These animals are also not used for  
2262 reproduction. Thus, although genotoxicity is a strong adverse effect, the biological  
2263 relevance for the target animals of this example (chicken for fattening) is limited.

**2264 Relevance for the consumer**

2265 For the assessment of the safety for the consumer, the critical question relates to  
2266 whether the carcinogen (estragole) is transferred to human food obtained from chickens  
2267 fed with the additive. Therefore, the ADME profile of the genotoxic compound has to be  
2268 investigated and analytical data of possible residues in edible tissues of the chicken are  
2269 needed, performed with methods being sensitive enough to detect very small  
2270 concentrations of the critical compound and its active metabolites. If the compound is not  
2271 absorbed or totally metabolised to innocuous compounds and if the absence of the  
2272 genotoxic compound itself or genotoxic metabolites thereof can be proven, the use of  
2273 the genotoxic compound in feeds may also be of no biological relevance for the  
2274 consumer. However, it is often difficult to demonstrate the absence of the genotoxic  
2275 compound or its metabolites in products derived from animals fed with the additive, for  
2276 technical reasons (the sensitivity of the analytical method applied results in an “analytical  
2277 threshold”). The addition of an essential oil containing estragole is therefore of biological  
2278 relevance for the consumer.

**2279 Relevance for the user**

2280 Genotoxic compounds in feed additives may create a concern for the safety of the user, if  
2281 any contact with such compounds cannot be avoided. Exposure to estragole while  
2282 handling the compound can occur mainly via skin contact and inhalation of the star anise  
2283 oil. The presence of estragole in feed of chicken for fattening represents a biologically  
2284 relevant hazard for users handling the additive, which is of biological relevance.

**2285 Relevance for the environment**

2286 Estragole is a naturally occurring compound in plants present in the European  
2287 environment. Because of the relatively low concentration in the feed of chickens for  
2288 fattening and the metabolism in the target animal, possible residues of estragole in the  
2289 excreta of the birds will not measurably increase the concentration of this compound in  
2290 the environment. For these reasons, the presence of estragole in the feed of chickens for  
2291 fattening is considered without biological relevance for the environment.

**2292 Overall Conclusion**

2293 During problem formulation, the presence of estragole was identified as a hazard  
2294 associated with the exposure to the additive, particularly for consumers potentially  
2295 exposed to residues of the additive via products of animal origin (meat) and users  
2296 exposed via inhalation. Considering that the intentional addition of compounds with  
2297 genotoxic-carcinogenic properties to the food chain via feed additives should be avoided  
2298 (minutes of the 109th Plenary meeting of the FEEDAP Panel), the applicant reformulated  
2299 the additive to remove estragole from the additive.

**2300 Uncertainties****2301 Adverse effects**

2302 The MoA for the genotoxicity of estragole is the oxidative conversion to 1-  
2303 hydroxyestragole, which is further conjugated with sulfate to the ultimate carcinogen  
2304 (Boberg et al., 1983). After long term treatment of mice the animals developed  
2305 significant increases in the incidence of hepatocarcinomas. The high sensitivity of mice to  
2306 develop liver cancer limits the extrapolation of this effect to humans. However, it was  
2307 demonstrated that the metabolism of estragole leading to DNA adducts in the liver is not



restricted to mice and occurs also in other species including birds and humans. The carcinogenicity of estragole in mice can therefore be taken as evidence for a possible carcinogenic effect of estragole in other species including humans.

It was demonstrated that the percentage of 1-hydroxyestragole formed after application of estragole to mice and rats increases with the administered dose. At low concentrations, estragole is mainly metabolized via alternative pathways to non-genotoxic compounds (Anthony et al., 1987). Taken into consideration that the concentration of estragole residues in the tissue of chicken treated with the additive is very low, this fact increases the uncertainty that estragole residues represent a biologically relevant hazard for the consumer.

Relevant adverse effects of trans-anethole and other non-genotoxic irritating compounds of the feed additive are restricted to the user/worker. These effects depend on the mode and level of exposure and thus to the safety precautions which are in place at working facilities.

#### Positive effect

The feed additive has the capacity to increase the performance of chicken for fattening, providing positive economic effect for the farmer. Any such effect exceeding the costs of the additive can be considered as relevant. However, the animal itself will not benefit from this positive effect.

Considerable uncertainty exists about whether such effects might be attributed to certain compounds of the feed additive or they rather reflect an additive effect of the mixture.

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- 2399

**Annex F – GMO**

**Scientific Opinion on application EFSA-GMO-NL-2007-45 for the placing on the market of herbicide-tolerant, high-oleic acid, genetically modified soybean 305423 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Pioneer**

**EFSA Journal 2013; 11 (12): 3499**

**Assessment strategy**

The EFSA GMO Panel was requested to carry out a scientific assessment of soybean 305423 (Unique Identifier DP-305423-1) for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003, i.e., to assess whether the import of Soybean 305423 and/or any of its derived products in the EU would result in additional safety concerns to animal and human health or to the environment with respect to conventional soybean.

This application excludes cultivation in the European Union and the Environmental Risk Assessment is limited to the consequences of accidental spillage of imported soybeans and to the dissemination of faeces of animals feeding Soybean 305423.

**Specification of the agent**

1) The GM plant itself: Soybean 305423

Soybean 305423 was transformed:

- to express the *Glycine max-hra (gm-hra)* gene conferring tolerance to acetolactate synthase (ALS)-inhibiting herbicides;
- to express a fragment of the endogenous *fad2-1* gene resulting, through RNA interference, in the silencing of the endogenous *fad2-1* gene, which leads to a decreased level of the omega-6 fatty acid desaturase and a high-oleic acid phenotype.

Soybean 305423 can be present through a wide range of genetic backgrounds into which the event has been introduced through backcrosses from the original transformed line.

2) Newly expressed protein: GM-HRA

Two forms of this protein are considered:

- a. The GM-HRA protein expressed in soybean 305423
- b. The equivalent GM-HRA protein expressed in a recombinant microbial system (*E. coli*) to be used in toxicological studies.

**Specification of the subjects**

- Humans who are exposed to the agents through the consumption of soybean 305423 oil or of food derived from soybean 305423;
- Animal species that are exposed to the agents through the consumption of soybean 305423 derived feed.

**Specification of the effects**

For GMOs, the risk assessment framework refers to the identification and characterisation of intended effects (those related to the new genes introduced into the plant) as well as

2441 unintended effects, i.e., all other changes that may result from the genetic  
2442 transformation.

2443 Soybean 305423, the genetic modification is intended to introduce two new traits  
2444 (intended effects of the genetic modification): herbicide tolerance and high-oleic acid  
2445 phenotype.

2446 Intended effects are known *a priori* to occur, while the size of their change might need  
2447 confirmation.

- 2448 - increase in oleic acid contents in soybean 305423 compared to conventional  
2449 soybean, which in turn might change the prevalence of oleic acid in the diets of  
2450 animals and humans;  
2451 - tolerance to ALS-inhibiting herbicides, likely to lead to a change of weed control  
2452 management of Soybean 305423, which in turn might lead to changes in plant  
2453 metabolism and the presence of ALS-inhibiting herbicides residues in the plant.  
2454

2455 Unintended effects are not known *a priori* and include:

- 2456 - changes in the agronomic and phenotypic characteristics (e.g., plant height, seed  
2457 weight) of soybean 305423 compared to its comparator, which may be indicative  
2458 of changes in the metabolism;  
2459 - changes in the level of endogenous components of soybean 305423 compared to  
2460 its comparator, which in turn may affect the nutritional balance of animal and  
2461 human diets or induce toxicological effects (dependent on the specific toxic  
2462 compounds whose level has changed);  
2463 - toxicological and allergenic effects of the newly expressed protein GM-HRA;  
2464 - increased allergenicity of Soybean 305423 compared to conventional counterpart  
2465 (*soybean is considered a common allergenic food [EC, 2007]*).  
2466 - presence of Open Reading Frames (ORFs), which might express peptides.  
2467

2468 Indirect and/or delayed effects may also result from the changes in agricultural practices  
2469 induced by the introduction of the GM plant but such effects are not relevant in the  
2470 context of this application that does not cover cultivation in the EU.  
2471

2472 The introduction of Soybean 503423 on the market (through the import and processing  
2473 of materials, beans and/or meals) could replace already used conventional soybeans in  
2474 animal feeding and human use.

2475 Under this scenario, the safety of soybean 503423 was assessed as regards its intended  
2476 trait (newly expressed protein and modified fatty acid profile) and unintended changes  
2477 observed.

2478 Considering the modified fatty acid profile of soybean 503423, the impact of this  
2479 replacement on the diet and in feedstuff formulation was assessed by an exposure  
2480 assessment. For the oil derived from soybean 503423 (the main product for human  
2481 consumption), a replacement dietary exposure assessment was performed to investigate  
2482 whether unbalanced diet for humans might result from soybean 503423 oil consumption,  
2483 including investigations on the changes in the level of fatty acids for which nutritional  
2484 recommendations exist.

2485 Possible impacts of changes in the level of endogenous toxic (anti nutritional) compounds  
2486 in soybean 503423 compared to conventional counterparts of relevant for food and feed  
2487 safety are assessed in the application.  
2488

2489 **Specification of the condition(s)**

2490 Conditions that should be implemented to assess the effects of the agents on the  
2491 subjects are described in guidance documents (EFSA, 2011).

2492 These include:

- 2493 - A set of field trials comparing the GM plant introduced in a specific genetic  
2494 background, its conventional counterpart (only differing from the GM plant by the  
2495 genetic transformation) and a range of commercial reference varieties (to  
2496 establish natural variability); field trials should be carried out under  
2497 representative receiving environments; agronomic, phenotypic and compositional  
2498 characteristics are measured and are subject to a difference and equivalence test;
- 2499 - Rodent studies on the newly expressed protein GM-HRA (standard, according to  
2500 OECD TG 407), if needed .
- 2501 - Rodent and broiler studies on the whole food feed from soybean 305423, as  
2502 needed (according to EFSA guidance or *ad hoc* protocol respectively)
- 2503 - Allergenicity testing on whole soybean extracts (human sera)
- 2504 - Dietary intake/exposure scenarios for intended changes in oleic acid (*ad hoc*  
2505 protocol)

2507 **Data collection**

2508 The risk assessment strategy for GM plants and derived food and feed proposed seeks to  
2509 deploy appropriate approaches to compare GM plants and derived food and feed with  
2510 their respective comparators. The underlying assumption of this comparative approach is  
2511 that traditionally cultivated crops have gained a history of safe use for consumers and/or  
2512 domesticated animals and the risk assessment primarily focused on new proteins and/or  
2513 changes in composition of the GM plant. The starting point of the data collection aims at  
2514 identifying similarities and differences between the GM plant and its conventional  
2515 counterpart (see above).

2516 Data were provided by the applicant in the form of dossier.

2517 Data provision was based on requirements by EFSA GMO guidance documents (EFSA  
2518 Guidance for risk assessment of food and feed from GM plants; EFSA ERA)

2519 *Ad hoc* additional data asked from EFSA and/or provided by the applicant. These were  
2520 necessary to corroborate and to further clarify information on:

- 2521 1) **Agents:** further molecular characterisation and details on RNA interference  
2522 mechanisms introduced by genetic modification for soybean 305423; structural  
2523 and functional characteristics; toxicological profile and allergenicity of newly  
2524 expressed protein GM-HRA.
- 2525 2) **Identification of the effects:** clarification on the outcome of comparative  
2526 analysis assessment (agronomic and phenotypic characteristics and particularly  
2527 compositional analysis) and possible biological effects (nutritional) of these on  
2528 consumers/animals
- 2529 3) **Identification of conditions:** comparator used in comparative assessment  
2530 studies; statistical methodology used in comparative assessment studies; test  
2531 conditions in toxicological and animal feeding studies; allergenicity testing of  
2532 soybean extracts on human sera; dietary exposure scenarios in humans and  
2533 animals;

2534



2535 Data included:

- 2536 • a molecular characterisation, which provides information on the structure and  
2537 expression of the insert(s) and on the stability of the intended trait(s);
- 2538 • a comparison, under representative field conditions, of agronomic, phenotypic and  
2539 compositional characteristics between the GM plant and its conventional  
2540 counterpart (field trials, EFSA Guidance)
- 2541 • a toxicological assessment, which addresses the impact of biologically relevant  
2542 change(s) in the GM plant and/or derived food and feed resulting from the genetic  
2543 modification; in this case on GM-HRA the assessment of potential allergenicity, of  
2544 the novel protein(s) as well as of the whole food derived from the GM plant *by*  
2545 *comparing the allergen repertoire with that of its appropriate conventional*  
2546 *counterpart(s)*
- 2547 • a nutritional assessment to evaluate whether food and feed derived from a GM  
2548 plant is not nutritionally disadvantageous to humans and/or animals, in particular  
2549 on fatty acid profile of soybean 305423.

## 2551 Data evaluation for each dataset

### 2552 Relevance of the agents

2553 **GM plant:** the soybean 305423 used in the assessment is relevant for the assessment,  
2554 this having been substantiated by data (sequence, expression, stability of inserts).

2555 The event was included in one specific genetic background which is a typical soybean  
2556 variety.

### 2557 Newly expressed protein: GM-HRA

2558 a. GM-HRA protein expressed in soybean 305423: fully characterised by experimental  
2559 data

2560 b. equivalent GM-HRA protein expressed in a recombinant microbial system (*E.coli*): fully  
2561 characterised by experimental data, equivalent to the plant protein and adequate for tox  
2562 studies.

### 2563 Relevance of the subjects

2564 Some limitations were identified:

2565 Humans: dietary intake and exposure scenario for edible oil were based on UK  
2566 population.

2567 Animals: Experimental animals were used (toxicological study on the new protein);  
2568 possible extrapolation to humans/other species could constitute an uncertainty).

### 2569 Relevance of the effects

2570 To go beyond the analysis of statistical differences between the GM plant and its  
2571 conventional counterpart and to put such differences into the context of the natural  
2572 variation of the measured endpoints among conventional soybean varieties, a test of  
2573 equivalence is carried out (see box). Effects were identified and considered relevant for  
2574 assessment, based on the outcomes of the difference/equivalence tests in the  
2575 comparative assessment and of the nutritional and toxicological studies.

2576



2577 **1. increase in oleic acid and MUFA in GM soybean compared to comparators**  
 2578 - *Is the effect in itself adverse/positive:* not adverse, might be beneficial

2579 - *is the effect essentially linked to an adverse outcome:* NO, these fatty acids are normal  
 2580 diet constituents;

2581 - *is it directly or indirectly linked to a(n) adverse/beneficial outcome?* Possible (dietary  
 2582 perturbations)

2583 - *Significant size of the effect:* YES (eg. oleic acid goes up from 20% to almost 80%)

2584 **RELEVANT FOR FURTHER ASSESSMENT → Exposure assessment of European**  
 2585 **populations based on scenarios (e.g., full replacement)**

2586

2587 **2. Decrease in n-6 PUFA (linoleic acid) in GM soybean compared to comparator**

2588 - *Is the effect in itself adverse/positive:* might be adverse

2589 - *is it directly or indirectly linked to a(n) adverse/beneficial outcome?* YES, deficiency in  
 2590 PUFA Linoleic acid (LA) is the main dietary n-6 PUFA in the human diet. EFSA has  
 2591 proposed an adequate intake (AI) for LA of 4 E %, based on the lowest estimated mean  
 2592 intakes of the various population groups from a number of European countries, where  
 2593 LA deficiency symptoms are not present. This AI corresponds to 9 g linoleic acid/day for  
 2594 an energy intake of 2 000 kcal.

2595 - *Significant size of the effect:* YES (e.g., for C18:2, the level decreases from 50% to less  
 2596 than 10%).

2597 **RELEVANT FOR FURTHER ASSESSMENT → Exposure assessment of European**  
 2598 **populations based on scenarios (e.g., full replacement))**

2599

2600 **3. Changes in odd fatty acid chain in GM soybean compared to comparator**

2601 - *Is the effect in itself adverse/positive:* Not adverse, these fatty acid are a normal diet  
 2602 constituent

2603 - *is it directly or indirectly linked to a(n) adverse/beneficial outcome?* Possible (dietary  
 2604 perturbations)

2605 - *Significant size of the effect:* YES (statistically).

2606 **RELEVANT FOR FURTHER ASSESSMENT → Exposure assessment of European**  
 2607 **populations based on scenarios (e.g., full replacement)**

2608

2609 **4. Changes in levels of calcium, zinc and glycitin and related total glycitein**  
 2610 **equivalents; trypsin inhibitor**

2611 - *Is the effect in itself adverse/positive:* Not adverse, these are a normal diet constituent

2612 - *is it directly or indirectly linked to a(n) adverse/beneficial outcome?* Possible

2613 - *Significant size of the effect:* YES from the statistical point of view (different and non-  
2614 equivalence demonstrated or more likely than not). However, these differences were not  
2615 considered biologically relevant for further safety assessment owing to their well-known  
2616 biochemical roles and to the magnitude of the reported levels.

2617 **NOT RELEVANT FOR FURTHER ASSESSMENT**

2618

2619 **5. Allergenicity of the newly expressed protein GMHRA: NO EFFECTS: no**  
2620 **indication that the protein is allergenic (source, bioinformatics etc)**

2621 - *Is the effect in itself adverse/positive: the potential effect could be negative;* not  
2622 applicable here

2623 - *is it directly or indirectly linked to a(n) adverse/beneficial outcome?* The potential effect  
2624 could be linked to an adverse outcome; not applicable here;

2625 - *Significant size of the effect:* not applicable here

2626 **NOT RELEVANT FOR FURTHER ASSESSMENT**

2627 **6. Increased allergenicity of the whole soybean 305423 plant compared to**  
2628 **conventional comparators: NO EFFECT: no change in 2-D immunoblot patterns;**  
2629 **no differences at ELISA analyses**

2630 - *Is the effect in itself adverse/positive:* the potential effect could be negative; not  
2631 applicable here

2632 - *is it directly or indirectly linked to a(n) adverse/beneficial outcome?* the potential effect  
2633 could be linked to an adverse outcome; not applicable here;

2634 - *Significant size of the effect:* not applicable here

2635 **NOT RELEVANT FOR FURTHER ASSESSMENT**

2636 The EFSA GMO Panel concludes that the composition of soybean 305423 differs from that  
2637 of the conventional counterpart and of non-GM reference varieties in its fatty acid profile,  
2638 the newly expressed protein, the minerals zinc and calcium and the isoflavone glycitin.  
2639 The variations in the fatty acid profile and the newly expressed protein are consistent  
2640 with the objective of the modification as well as with the expression of the ALS enzyme of  
2641 soybean 305423. A safety and nutritional assessment of the altered fatty acid profile and  
2642 the newly expressed protein is provided in section 5 of the EFSA Scientific Opinion. For  
2643 the remaining compounds, no further assessment was deemed necessary owing to their  
2644 well-known biochemical roles and to the magnitude of the reported levels.

2645 **Relevance of the conditions**

2646 Field trials: in accordance to EFSA 2011

2647 Toxicological studies: compliant with standard OECD protocol

2648 Animal feeding studies: ad hoc protocols, not standardised; considered adequate by the  
2649 EFSA GMO panel

2650 Exposure assessment: ad hoc study protocol used. considered adequate by the EFSA  
2651 GMO panel.

## 2652 Uncertainties

2653 1. General assumption for comparative assessment:  
2654 The underlying assumption of this comparative approach is that traditionally  
2655 cultivated crops have gained a history of safe use for consumers and/or  
2656 domesticated animals.

2657 2. Use of the microbial protein as a surrogate of the plant protein:  
2658 The *E.coli*-derived GMHRA protein was fully characterised by experimental data,  
2659 found to be similar to the plant protein and considered adequate for toxicological  
2660 studies; however some differences were identified between the microbial  
2661 surrogate protein and the plant protein (the purification process of the microbial  
2662 protein included the cleavage of the His-tag with thrombin; the resulting microbial  
2663 GM-HRA protein has an additional glycine residue at the N-terminus compared  
2664 with the mature GM-HRA protein expressed in soybean 305423 leaves). Not  
2665 considered limitative, just noted in the scientific opinion.

2666 3. Exposure scenarios for fatty acids:  
2667 4. based on UK database only, not representative of the whole EU population. This  
2668 was considered relevant and it was suggested, in the post-market monitoring  
2669 (PMM) to focus on the collection of consumption data for the European population.  
2670

## 2671 Use of statistical equivalence testing

2672 Equivalence testing is currently being used in the safety assessment of GM plants (EFSA,  
2673 2011). Indeed, in many cases, the starting point of the risk assessment consists in  
2674 comparing the biological system exposed to the agent with the same biological system  
2675 not exposed (e.g., dose-mortality response of test species to a pesticide, a chemical or a  
2676 recombinant protein, compositional analysis of a GM plant in comparison with its  
2677 conventional counterpart performed across a range of field studies). The comparison  
2678 begins by measuring a number of specific endpoints and an assessment as to whether  
2679 the exposed biological system is different from its « conventional counterpart». This is  
2680 usually done through a test of difference that leads to a list of significant statistical  
2681 differences.

2682 However, statistical difference does not necessarily mean that the difference/effect is  
2683 biologically relevant and the observed differences need to be put in the context of the  
2684 natural variation of the biological system non-exposed to the agent (i.e., observed under  
2685 different conditions). Indeed, many environmental or biological factors may affect the  
2686 natural variation of the biological system non exposed to the agent.

2687 In this context, equivalence is defined as the absence of differences other than ordinary  
2688 biological variation. For each chosen endpoint, or for groups of endpoints, limiting values  
2689 for which the difference is acceptable, must be determined. These are known as  
2690 equivalence limits.

2691 When historical data on the natural variation of the biological system are available, it is  
2692 possible to establish equivalence limits prior to the comparative risk assessment.  
2693 Otherwise, natural variation of biological system could be estimated in the same studies  
2694 as those carried out to assess the effect of the agent on the biological system. This is

done by measuring endpoints on subjects other than those used to test the agent. For example, in the GMO comparative analysis, commercial varieties are included in the field experiments, together with the GMO under assessment and its direct conventional counterpart. Statistical methods can be used to assess the observed differences against the natural variability observed among subjects not exposed to the agent. (Cf Statistical Guidance Document).

Both difference and equivalence testing approaches are complementary: statistically significant differences may point at direct biological changes caused by the GM transformation, but that may not be relevant from the viewpoint of safety. On the other hand, equivalence assessments may identify differences that are potentially larger than normal natural variation. It should be pointed out that even if a difference is proven to fall within natural variation, it might still be relevant for further toxicological assessment if this change is observed consistently across subjects or if it may lead to indirect/carryover effects on the functioning of the biological system/ecosystem.

## References

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2013. Scientific Opinion on application EFSA-GMO-NL-2007-45 for the placing on the market of herbicide-tolerant, high-oleic acid, genetically modified soybean 305423 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Pioneer. EFSA Journal 2013;11(12):3499, 35 pp. doi: 10.2903/j.efsa.2013.3499

## Annex G – NDA

**Case study of biological relevance in the area of health claims: Application for substantiation of a health claim related to: Yestimun® and defence against pathogens in the upper respiratory tract (EFSA Journal 2013;11(4):3159)**

### Assessment strategy

$\beta$ -glucans are dietary fibres that have been shown to have immunomodulatory activity in animals and humans after oral administration. Common colds are caused by viruses, which are pathogens that are eliminated by the body's defence mechanisms. By virtue of the effect of  $\beta$ -glucans on the immune system, the fibres may support defence against pathogens in the upper respiratory tract.

The food, which was the subject of the claim, that the NDA Panel was requested to evaluate, was Yestimun®, i.e. (1,3)-(1,6)- $\beta$ -D-glucan produced from brewer's yeast cell wall (100 % *Saccharomyces cerevisiae*), and the claim was that this food would support the defence against pathogens in the upper respiratory tract.

The NDA Panel needed to judge the claimed effect according a number of criteria:

1. Specification of the agent: is the food sufficiently characterized to evaluate the claimed effect
2. Specification of the subjects: Which is the target group for the claimed effect, and
3. Identification: Is the claimed effect in itself a relevant health effect, i.e. is it biologically relevant, and
4. Specification of the conditions: Is the information based on human intervention studies and other information. Are the studies, on which the applicant wants to base the claim of glucans supporting defence to respiratory infection, sufficiently powered and are measures performed according accepted standards and is statistical analysis done appropriately. As long as common cold could be attributed to infection with an infectious agent, such information would be useful. Information on other respiratory conditions that are not attributable to infection is not useful. Information on immune parameters, assessed in humans, animals, or in vitro systems, can only provide supportive or mechanistic information, but in the absence of a substantiated effect on defence do not provide any scientific evidence for the substantiation of the claim. Does a biological effect occur after ingestion of the glucan; reduction of an already evident infection is beyond the scope of health claims on food, such effects would be considered as therapeutic.

### Data Collection

For the substantiation of the claim, three randomized controlled intervention studies were available. The primary endpoints were reduction in the number of common cold episodes per subject during the study periods, whereas secondary outcomes were severity of common cold episodes, duration of cold episode and the use of antibiotics and analgesics. The applicant provided information on the incidence of common cold.

### Data Evaluation

The NDA Panel considered that the food was sufficiently characterized for the purpose of evaluating the claimed effect.

In addition, the NDA Panel considers that supporting defence against pathogens in the upper respiratory tract, as measured by episodes of common cold, is in itself a beneficial physiological effect and therefore biologically relevant. In order to substantiate the claim, a statistically significant decrease in common cold episodes, if adequately proven with adequate confidence limits, would have been sufficient to substantiate the claim.

For the substantiation of health claims, the NDA Panel requires human data, notably intervention studies in humans. The target population is the general population and the subjects in the study, i.e. healthy individuals, represent the target population.

The evidence provided did not establish the validity of questionnaires and criteria used to assess the incidence or the severity of common cold episodes. The power of the studies were likely adequate to observe effects on the primary endpoints. However, in one of the studies post-hoc analyses were performed based on episodes that occurred in the winter months (November to March, first half of the study) to avoid the potential error that might have arisen owing to possible misdiagnosis of allergic rhinitis as common cold infections during the summer months. Whereas this notion is understood, the post-hoc selection of the time windows for calculation of possible effects was not accepted as valid. In another study, statistical analysis did not account for the multi-centre design of the study.

Overall conclusion

In the judgement of the Panel, an effect on incidences of common cold, if appropriately shown to be statistically significant, would have been relevant for the purpose of substantiating the claim. However, even if statistically significant differences were reported, they were not judged relevant due to flaws in the statistical approach and the Panel came to the conclusion that a cause and effect relationship had not been shown.

### Uncertainty

On the basis of the data presented, the NDA Panel concluded that a cause and effect relationship had not been established between the consumption of Yestimun® ((1,3)-(1,6)- $\beta$ -D-glucans from brewer's yeast cell wall) and defence against pathogens in the upper respiratory tract. The opinion did not indicate that such a relation could not be there, but indicated that from the human studies provided conclusions could not be drawn.

### References

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2013. Scientific Opinion on the substantiation of a health claim related to Yestimun® and defence against pathogens in the upper respiratory tract pursuant to Article 13(5) of Regulation (EC) No 1924/2006. EFSA Journal 2013; 11(4): 3159, 12 pp. doi:10.2903/j.efsa.2013.3159.



## 2802 Dietary Reference Values

### 2803 Introduction

2804 Following a request from the European Commission, the EFSA Panel on dietetic products,  
2805 nutrition and allergies (NDA) was asked to deliver a scientific opinion on dietary  
2806 references values (DRV) for vitamin D for the European population.

2807 Vitamin D is a generic term for ergocalciferol (vitamin D<sub>2</sub>) and cholecalciferol (vitamin  
2808 D<sub>3</sub>), which are formed from their respective provitamins, ergosterol and 7-  
2809 dehydrocholesterol, following a two-step reaction involving ultraviolet B (UV-B)  
2810 irradiation and subsequent thermal isomerisation. Vitamin D<sub>2</sub> and vitamin D<sub>3</sub> are present  
2811 in foods and dietary supplements. Vitamin D<sub>3</sub> is also synthesised endogenously in the  
2812 skin following exposure to UV-B. However, the properties of sunlight in Europe are not  
2813 sufficient for vitamin D<sub>3</sub> synthesis during several months each year, resulting in the so  
2814 called vitamin D winter.

2815 Vitamin D deficiency leads to impaired mineralisation of bone due to an inefficient  
2816 absorption of dietary calcium and phosphorus, and is associated with an increase in  
2817 parathormone concentration. Clinical symptoms of vitamin D deficiency manifest as  
2818 rickets in children and osteomalacia in adults.

### 2819 Assessment strategy

2820 A balanced diet is one that provides adequate amounts of energy and nutrients for health  
2821 and well-being. DRVs comprise a complete set of nutrient reference values, such as lower  
2822 threshold intake (LTI), average requirement (AR), population reference intakes (PRI),  
2823 average intake (AI), and tolerable upper intake levels (UL).

2824 PRIs can be used for instance as a basis for reference values for food labelling, or for  
2825 establishing food-based dietary guidelines (FBDG). FBDG translate nutritional reference  
2826 values into messages about foods and diet, which can guide consumers on to what to eat  
2827 in order to fulfil their nutritional requirements.

2828 Thus, DRVs on vitamin D should ensure that the corresponding requirements be covered  
2829 in the European population, without achieving any toxic effect.

### 2830 Relevance of the evidence/data

2831 Ideally, nutritional requirements are measured in a subset of the target population, for  
2832 instance using balance studies to assess the exact amount of a given nutrient, which  
2833 should be consumed daily by each individual to offset losses and maintain stores at their  
2834 optimal level. These data allow defining an AR. Taking AR variance into account, it is  
2835 possible to calculate a PRI, i.e. a level of intake which should cover the requirements of  
2836 97.5% of the population.

2837 In this instance, the Panel considered that available data did not allow defining an AR,  
2838 hence calculating a PRI. Instead, the Panel chose to set an AI.

2839 In a first step, the Panel searched for biomarker of vitamin D status. Intervention and  
2840 prospective observational studies were considered using endpoints related to  
2841 musculoskeletal health through bone measurements (BMC, BMD) obtained via different  
2842 techniques and after an appropriate study duration (e.g. at least one year), as well as  
2843 the assessment of osteomalacia or bone fractures. Other health outcomes were also  
2844 considered, such as adverse pregnancy-related outcomes, but the example is restricted  
2845 to adult males and non-pregnant females.

2846 Although the results were somewhat blurred by the use of different analytical methods, it  
2847 was possible to conclude that there is evidence for an increased risk of adverse  
2848 musculoskeletal health outcomes at serum 25(OH) concentrations below 50 nmol/l. Thus,  
2849 the serum concentration of 25(OH)D can be considered as a surrogate marker of vitamin  
2850 D status.

### 2851 Nature and size of the effect

The next step was to assess the relationships between 25(OH)D and vitamin D intakes. In order to avoid confounding by endogenous synthesis, studies carried out during minimal sun exposure, lasting for at least 6 weeks, with oral exposure to vitamin D at least twice a week were considered. The articles which matched eligibility criteria were used to perform a quantitative analysis of the extracted data through a meta-analytic approach. Background intake was added to the supplemental vitamin D dose to generate total vitamin D estimates. When the habitual vitamin D intake was not reported, surrogates were imputed using appropriate age- and sex-specific mean vitamin D intake values (from food) from the national nutrition survey relevant to the country in which the study was performed.

Two different models of the dose-response relationship between total vitamin D intake and plasma/serum 25(OH)D concentration were explored : a linear model and a non-linear model (i.e. with the natural logarithm transformation of the total intake). Finally, the Panel decided to retain the non-linear model to better describe the dose-response shape and to be able to include results from higher dose trials (i.e up to 50 µg/day).

A number of factors potentially influencing the dose-response relationship were investigated, in order to select factors to be included in the final model to characterise the high heterogeneity of results across individual trials. After the inclusion of the final set of covariates, the adjusted  $R^2$  (proportion of between-study variance explained) of the final model was 85 %, meaning that the fitted factors were able to characterise most of the across-trials variability in response. The models were used to predict the achieved mean serum 25(OH)D concentrations corresponding to total vitamin D intakes of 5, 10, 15, 20, 50, 100 µg/day and to estimate the total vitamin D intakes that would achieve serum 25(OH)D concentrations of 50, 40, 30, 25 nmol/l.

A number of sensitivity analyses were also carried out to evaluate whether the findings were robust to the assumptions made in the systematic review protocol and the analyses, in particular, on the background intake imputation process, on eligibility criteria (e.g. fortified food trials versus supplement trials); characteristics of participants (e.g. exclusion trials that did not explicitly exclude supplement users, persons with sun holidays, persons using sunbeds/artificial UV-B sources or going on sunny holidays). None of these sensitivity analyses raised serious concerns about the robustness of the overall analysis.

The Panel considered that the results of this meta-regression analysis could be used to set DRVs for vitamin D.

#### **Overall relevance taking into account the exposure**

The Panel used information obtained from characterising the intake-status relationship for vitamin D to derive the vitamin D intake to achieve a target serum 25(OH)D concentration of 50 nmol/l. For the purpose of deriving AIs for vitamin D, the Panel decided to focus on the *adjusted* model obtained with data mostly on adults. The estimates from that model were derived based on all covariates.

In the *adjusted model*, the total intake estimated to achieve a serum 25(OH)D concentration of 50 nmol/l, as identified by the lower limit of the 95% PI, is 16.1 µg/day. Equally, at a vitamin D intake of 15 µg/day, the predicted mean serum 25(OH)D concentration is 63 nmol/l (95 % CI: 58–69 nmol/l), with a predicted value at the lower limit of the 95% PI of 49 nmol/l.

Predicted interval (PI) in the context of a meta-regression analysis illustrates the uncertainty about the true mean response predicted in a future study. Moreover, 95% PI constitutes an approximation of the interval that would include 95% of all individual responses from the populations included in previous and future studies, as it refers to the population of mean responses. The extent of this approximation could not be quantified.

The Panel therefore set an AI for vitamin D for adults at 15 µg/day, considering that, at this intake, the majority of the adult population will achieve the target serum 25(OH)D concentration near or above 50 nmol/L. The Panel decided not to set specific AIs for 'younger' or 'older' adults, because there was no evidence of a significant difference in absorption capacity between 'younger' and 'older' adults and the majority of the studies used to set the target value for 25(OH)D concentration were carried out in 'older adults'.

The unadjusted model can be also taken into account as it encompasses the whole heterogeneity across trials. In the unadjusted model, considering a vitamin D intake of 15 µg/day, the lower limit of the 95% PI is 34 nmol/L. This value is above the concentrations that have been observed in relation to overt adverse health outcomes (osteomalacia, calcium absorption). Considering a vitamin D intake of 15 µg/day, the upper limit of the 95% PI is 91 nmol/L in the unadjusted model (and 78 nmol/L in the adjusted model). These values are in the physiological range.

## References

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2016. Scientific opinion on dietary reference values for vitamin D. EFSA Journal 2016;14(10):4547, 145 pp. doi: 10.2903/j.efsa.2016.4547

## 2919 Annex H – PLH

2920 The EU plant health legislation aims to protect crops, fruits, vegetables, flowers,  
2921 ornamentals and forests from harmful pests and diseases (harmful organisms) by  
2922 preventing their introduction into the EU or their spread within the EU. This aim helps to:

- 2923 • contribute to sustainable agricultural and horticultural production through plant  
2924 health protection;
- 2925 • contribute to the protection of public and private green spaces, forests and the  
2926 natural landscape.

2927 The Council Directive 2000/29/EC<sup>2</sup> provides the basis for this aim. The general principles  
2928 are based upon provisions laid down in the International Plant Protection Convention  
2929 (IPPC, 2007).

2930 Directive 2000/29/EC is supported by further legislation in the form of a number of  
2931 Control Directives and Emergency Measures.

2932 In order to meet the aims of the regulation, the EU:

- 2933 • regulates the introduction of plants and plant products into the EU from countries  
2934 outside the EU;
- 2935 • regulates the movement of plants and plant products within the EU;
- 2936 • imposes eradication and containment measures in case of outbreaks, and co-  
2937 finances them;
- 2938 • places obligations on countries outside the EU which want to export plants or  
2939 plant products to the EU.

2940 The Panel on Plant Health (PLH) provides independent scientific advice on the risk posed  
2941 by plant pests which can cause harm to plants, plant products or biodiversity in the EU.  
2942 The Panel reviews and assesses those risks with regard to the safety and security of the  
2943 food chain.

2944 The EFSA plant health panel supports commission decisions on plant health by making  
2945 scientifically-based pest risk assessments. The risk assessment follow the structure  
2946 agreed by the IPPC (2007). The PLH panel has outlined its procedures in guidance  
2947 documents on the pest risk assessment (EFSA PLH panel, 2010) and the evaluation of  
2948 risk reduction options (EFSA PLH panel, 2012). The panel is currently working on a  
2949 framework for a risk analysis that is quantitative. Aims of introducing a quantitative  
2950 approach are to increase consistency, transparency and objectivity. In this new  
2951 approach, the steps in the assessment are elaborated quantitatively. The step are: (1)  
2952 pest entry into the EU, (2) pest establishment in the EU, (3) pest spread within the EU,  
2953 and (4) impact assessment. These steps are cumulative, as there will be no impacts  
2954 without the previous steps taking place. When compared to the risk assessment for toxic  
2955 or beneficial compounds, the first three steps are similar to exposure, while the impact  
2956 assessment has similarity to the dose-response relationship in toxicological studies.  
2957 Indeed, Robinet et al. (2016) use the term “exposure” to describe the contact rate of  
2958 native European trees with propagules of the pathogenic fungus *Ceratocystis fagacearum*  
2959 that can enter Europe on wood imported from the United States.

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<sup>2</sup> <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2000:169:0001:0112:EN:PDF>

2960 The current example is based on an ongoing risk assessment on the potato rot  
2961 nematode, *Ditylenchus destructor*. This risk assessment was elaborated following the  
2962 new quantitative approach. The opinion is in preparation for possible adoption by the  
2963 EFSA plant health panel in September 2016.

2964 In the pest risk assessment on *Ditylenchus destructor*, the panel focused the assessment  
2965 of entry, establishment, spread and impact on two crop species: potato (*Solanum*  
2966 *tuberosum*) and tulip (*Tulipa* spp.). The choice of these two species was based on  
2967 considerations of relevance as potato and tulip were judged, based on production areas  
2968 and trade flows, to be the most relevant pathways for introduction and spread of this  
2969 nematode with planting material, and also for the materialization of impacts in crop  
2970 production. Other flower bulb species could also be vectors for entry and spread, but  
2971 they were not considered because the trade volumes are much smaller than those of  
2972 tulips. Impacts in other bulbs species are also expected to be much smaller than those  
2973 that could occur in tulips because of smaller production areas.

2974 A modelling approach was used to estimate entry, spread and impact quantitatively.  
2975 Trade data were used for assessing import volumes. Literature and expert judgement  
2976 were used to estimate model parameters, taking into account uncertainty. Special  
2977 attention was paid to the evaluation of risk reduction options for planting material,  
2978 treatment of flower bulbs before trading, and treatment of soil prior to planting of potato.

2979 A baseline scenario with current pest-specific phytosanitary regulations was compared  
2980 with alternative scenarios without those specific regulations or with additional risk  
2981 reduction options. Further information was provided on the host range of *D. destructor*  
2982 and on survival of the pest in soil in the absence of hosts.

2983 The Panel concluded that the entry of *D. destructor* with planting material from third  
2984 countries is quite small compared to the yearly intra-EU spread of this nematode with  
2985 planting material. Changes in pest specific regulations have little influence on entry of the  
2986 pest. It was also concluded that the whole pest risk assessment area is suitable for  
2987 establishment of *D. destructor*, but there is insufficient information to make a statement  
2988 on the persistence of newly introduced populations. Impacts of this nematode on the  
2989 quantity and quality of potato production are considered negligible. The Panel also  
2990 considers the impact of this nematode on flower bulb production in the EU as very low.

## 2991 **Assessment strategy**

2992 The commission asked EFSA to assess various aspects of the risks of the potato rot  
2993 nematode *Ditylenchus destructor* to agriculture in the EU. The PLH panel decided to  
2994 conduct a pest risk assessment according to the framework provided by IPPC (2007),  
2995 entailing an assessment of the risks of entry, establishment, spread and impact. The  
2996 nematode is already present within the EU, albeit sporadically, and it is not entirely clear  
2997 *a priori* whether additional entry is of relevance for the impacts of this nematode. The  
2998 panel developed a model for entry, establishment, spread and impact to assess the  
2999 relative importance of entry of this nematode with trade from third countries and its  
3000 spread within Europe in intra-European trade of plant products.

## 3001 **Evidence/data needed to address the question**

3002 To assess this question, information is needed on trade flows in plant products that can  
3003 serve as a vector. Both the trade-flows from third countries (i.c. Canada and Switzerland



that have presence of this nematode but can import under certain restrictions) are considered, and those within Europe. Furthermore, information is needed on the prevalence of the nematode in the trade flows. Furthermore, information is needed on the host plants of the nematode, and the impact caused on those hosts. In addition information is needed on the conditions for establishment. The panel focused the assessment on seed potato and flower bulbs, which constitute the most important plant product that can serve as a vector. Furthermore, ware potatoes and many flower bulb species are hosts and can suffer damage.

#### Data evaluation

Adequate data are available on the international trade in seed potatoes. However, no adequate data are available on the trade in flower bulbs because the data in Eurostat are not recorded at a sufficient level of resolution between species. Therefore, trade in host species and non-host species cannot be well distinguished. Data on production areas of seed potatoes across the EU are good. Data on production areas of flower bulbs are adequate.

Information on the prevalence of the nematode in the EU and in third countries is extremely sparse. Only vague descriptions are available like "present in all parts of the area where host crops are grown", "present, restricted distribution", "present, few occurrences", "present", and "absent". These terms were interpreted by the panel in terms of proportion of production fields infested with the nematode and proportion of planting material harvested from infested fields that carry the nematode. This interpretation is a reason for large uncertainties in the estimates of the flow of infested planting material. Furthermore, the panel made assessments of survival of the nematode in trade flows, and of the efficacy of import and export inspection and of certification schemes to reduce or limit the levels of infestation of plant product with the nematode. Due to lack of pertinent data, these assessments were also quite uncertain.

#### Relevance of the agent

The potato rot nematode *Ditylenchus destructor* causes rots in root crops and bulbous crops. The species is well characterized and the potential damaging effects are also well characterized. If uncontrolled, this nematode can multiply, spread and cause substantial damage. However, with current phytosanitary measures for containing the spread with plant products, and efficient weed control in crops, reducing the number of potential hosts for the nematode, the impact of this nematode under current conditions is minor.

Nematodes can live in tubers, bulbs and rhizomes, and they can be spread with trade in such products over practically unlimited distance. Infested planting material is the main pathway for spread. Autonomous spread by nematodes is not practically relevant. Spread by farm machinery is possible but is still a short distance spread (mostly within field or farm). There is no known minimum number of nematodes that is required to cause establishment or infestation of a plant.

#### Relevance of the subject

The panel made the assessment focusing on seed potatoes and tulip bulbs. Seed potatoes are the most important carrier for the nematode. Flower bulbs are also potentially important, and the trade in the host species tulip is the largest amongst flower bulbs. Both potato and tulip suffer damage if infested.



3048 **Relevance of the effect**

3049 This nematode can cause considerable damage if there are not measures in place to  
3050 prevent this damage.

3051 **Relevance of the conditions**

3052 The assessment was carried out considering common practices in current trade and crop  
3053 cultivation in Europe. These conditions are fully relevant.

3054 **Overall conclusion**

3055 This nematode is present in two thirds of the EU member states, but is currently of minor  
3056 importance in Europe as current measures and agricultural practices are effective in  
3057 limiting spread and impact. Lifting pest-specific measures is not expected to change this  
3058 because certification of planting material of potato and flower bulbs needs to meet  
3059 quality criteria that would effectively limit the presence of this agent.

3060 **Uncertainty**

3061 The key uncertainty in the assessment is the current distribution. There are no reports on  
3062 structured surveys in the EU or in third countries to quantify at relevant spatial scales the  
3063 prevalence of this nematode. There is thus no relevant information available on: (1)  
3064 presence in geographic areas below the level of member state, (2) proportion of infested  
3065 area fields in those geographic areas in which the nematode occurs, and (3) proportion  
3066 of infested planting material harvested from infested fields. The lack of quantitative  
3067 information on the multi-scale presence of the organism is a great impediment to making  
3068 the assessment. Instead of basing its parameter estimates for the model on data, the  
3069 panel had to resort to expert judgements. In the assessment, the panel made use of  
3070 stochastic simulations, and the resulting distributions of outcomes show variability over  
3071 four orders of magnitude.

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## Annex I – PPR

### PESTICIDES HAVING EFFECTS ON THE THYROID HORMONE SYSTEM

In the MRL regulation 396/2005 it was laid down that account should be taken of “the possible presence of pesticide residues arising from sources other than current plant protection uses of active substances, and their known cumulative and synergistic effects, when the methods to assess such effects are available”.

Then in the later pesticide regulation 1107/2009, the precautionary principle applies and therefore before placing active substances in plant protection products on the market it should be demonstrated that they do not have any harmful effect on humans.

However, not only the single active substance should not have harmful effects but should also take account of effects from mixtures of pesticides. Thus, the regulation reads “shall have no immediate or delayed harmful effect on human health, including that of vulnerable groups, or animal health, directly or through drinking water (taking into account substances resulting from water treatment), food, feed or air, or consequences in the workplace or through other indirect effects, taking into account known cumulative and synergistic effects...”

This would be applicable not only for dietary risk assessment but also non-dietary (operators, workers, bystanders and residents).

EFSA was in accordance with the regulation commissioned to develop the methodology for carrying out cumulative risk assessment in regard to MRL-setting and launched this work in 2006 with a scientific colloquium followed by subsequent opinions, amongst the “Scientific opinion on the identification of pesticides to be included in cumulative assessment groups on the basis of their toxicological profile”. In the opinion a methodology for grouping was developed and two cases were elaborated on; Pesticides having effects on the nervous system and pesticides having effects on the thyroid system. A total of nearly 300 pesticide dossiers were evaluated for these two cases.

#### Assessment strategy

In this case, two problem formulations are being answered on the basis of the same data-sets.

1. For the single pesticidal active substance assessment, establish no observed *adverse effect levels* (NOAELs) in case of effects on the thyroid system.
2. Identification of pesticides to be included in cumulative assessment groups (CAG’s) on the basis of their toxicological profile (hazard assessment) – in this case effects on the thyroid system – and establishing NO(A)EL’s in this context.

The establishment of the critical effect, the NOAEL for this effect and deriving reference doses (AOEL, ADI, ARfD) is to protect the human population exposed to the pesticide when applied and to protect the population being exposed via all routes of exposure.

The grouping of the pesticides into CAG’s was developed to support the regulatory MRL-setting and as such the target population is the European Consumer.

Agents	Effects	Subjects	Conditions
Single active substance	Effects on the thyroid system	The human population	Dietary and non-dietary exposure. Establishment of NOAEL in subchronic – chronic exposure.
Cumulative Assessment Groups of active substances (CAGs)		The human population	Dietary exposure. Establishment of NO(A)EL in subchronic – chronic exposure.

3133

#### 3134 Data collection and selection of the biologically relevant data

3135 For the standard assessment of pesticidal active substances the regulation specifies  
 3136 extensive data requirements in regard to mammalian toxicity (exposure usually by the  
 3137 oral route) on several species and exposure duration (from sub-acute to chronic).

3138 In addition to the regulatory studies, where data from the scientific literature on  
 3139 pesticides and their effects on the thyroid system were available this was taken into  
 3140 account to support mechanistic understanding of the effect.

3141 No specific data requirements are set in the regulations for the purpose of grouping of  
 3142 pesticides into CAG's.

3143 For assessment of the histopathological findings, these are generally classified according  
 3144 to qualitative criteria and the data are presented as number of animals affected within a  
 3145 dose group. Numerical results, should according to the relevant OECD guideline, be  
 3146 "evaluated by an appropriate and generally acceptable statistical method". As the data  
 3147 base for grouping of pesticides for having effects on the thyroid system consists of a little  
 3148 less than 300 pesticide dossiers, the statistical methods applied will of course be different  
 3149 from study to study. But in all cases they have been assessed and peer-reviewed and  
 3150 NOAEL's have been established for the single substance evaluation.

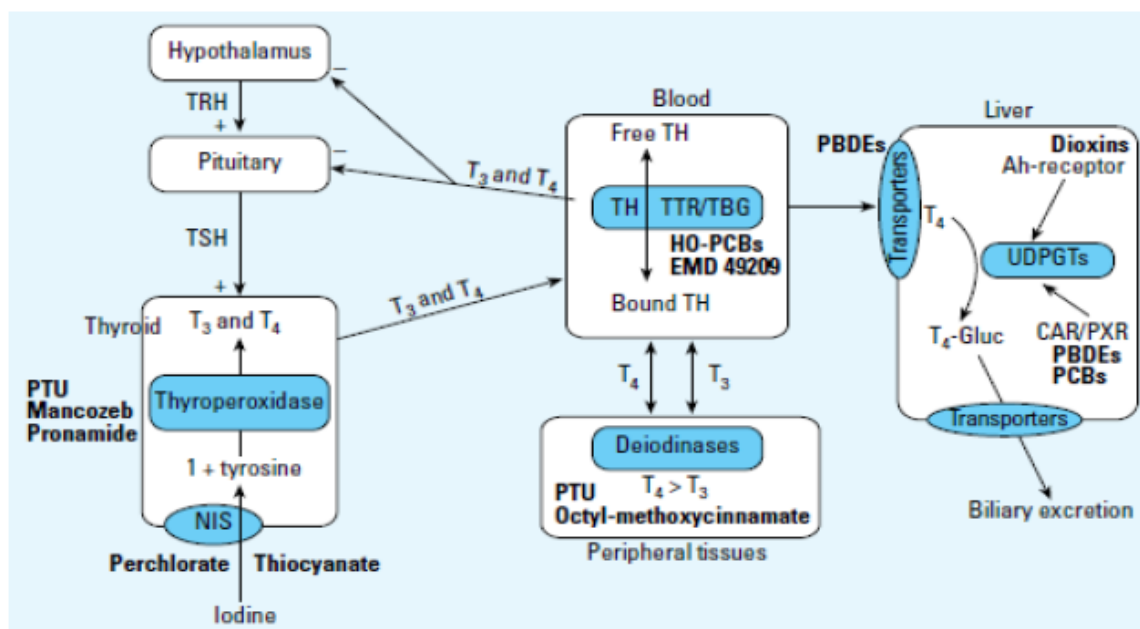
3151 The power of the studies would also be very varied going from the very low-powered dog  
 3152 study, with usually only 4 animals/sex/group, to more well-powered rodent students with  
 3153 20 animals/sex/group, and in case of carcinogenic effects, there are 50  
 3154 animals/sex/group. Normally the power of a given study for the different endpoints  
 3155 investigated is not stated.

3156 On studies in dog, results that are not statistically significant are also be considered for  
 3157 their biological significance, and individual values are been taken into account. It is

considered that the statistical significance in the dog studies might not be reliable due to the high inter-individual variability.

## Relevance of the effects

For detailed description of the thyroid system, see Miller (2009) and the following figure showing where chemicals might perturbate the thyroid system.



**Figure 1:** Thyroid hormone system with potential targets for disruption by xenobiotics (blue). NIS, sodium/iodide symporter; TBG, thyroid hormone-binding globulin; TH, thyroid hormone (T<sub>3</sub>/T<sub>4</sub>); T<sub>3</sub>, triiodothyronine; T<sub>4</sub>, thyroxine; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; TTR, transthyretin; UDPGTs, UDP-glucuronosyl transferases (Miller et al., 2009).

When declines in circulating and tissue hormone levels occur, feed-back mechanisms of the hypothalamic-pituitary-thyroid axis would result in increased secretion of TSH (Thyroid Stimulating Hormone) and subsequently the follicular cells would increase the secretion of T<sub>3</sub>/T<sub>4</sub> and thus levels of bio-available T<sub>3</sub>/T<sub>4</sub> would be re-adjusted.

## Relevance of thyroid system disruption to humans

Alterations in circulating bioavailable thyroid hormone levels may have serious impact on other organs or organ systems besides the thyroid itself also in humans, particularly if perturbations occur during critical windows of development" (EFSA 2013).

And further the PPR panel noted that "Any degree of thyroid disruption that lowers TH levels on a population basis should be considered a biomarker of increased risk of adverse outcomes, which may have important societal outcomes" (Miller et al., 2009) (EFSA 2013)". So as such adverse effects/effects on the thyroid system are considered relevant for the human population.

3179 **Is the effect in itself a(n) adverse/positive effect?**

3180 Single pesticide evaluation of adverse effects on the thyroid hormone system

3181 When evaluating adverse effect on the thyroid, physiological changes preceding adverse  
3182 manifestations in target organs (changes in circulating thyroid hormone levels) and  
3183 indicators of perturbation of thyroid hormone homeostasis (e.g. elevation of TSH based  
3184 or thyroid enlargement) would not be regarded as adverse when establishing the NOAEL  
3185 for thyroid effects in a study. The assumption is that as a consequence of changes in  
3186 circulating and tissue thyroid hormone levels, compensatory mechanisms including  
3187 activation of the hypothalamic-pituitary-thyroid axis following a decline in peripheral  
3188 thyroid hormone levels with subsequent increased production and secretion of TSH  
3189 (thyroid stimulating hormone) may be expected to result in adjustment of bioavailable  
3190 thyroid hormone levels. Thus, changes in circulating or tissue T3/T4 hormone levels  
3191 would be transient (EFSA 2013).

3192 Effects on the thyroid hormone system in regard to grouping for CRA

3193 In regard to grouping of pesticides for cumulative risk assessment other considerations  
3194 were also taken into account. It was noted that: "For the evaluation of the common  
3195 toxicological profile for assignment of an active substance to a CAG, different indicators  
3196 may be taken into account, which could comprise downstream endpoints with obviously  
3197 adverse target organ effects or upstream precursor effects e.g. a decrease in T4 levels,  
3198 that may eventually lead to manifestation of an adverse organ effect.

3199 In the context of CRA, it is therefore proposed to also consider the physiological change  
3200 preceding adverse manifestations in target organs (changes in circulating thyroid  
3201 hormone levels) and indicators of perturbation of thyroid hormone homeostasis (e.g.  
3202 elevation of TSH or thyroid enlargement), to be of relevance for definition of cumulative  
3203 assessment groups." (EFSA 2013)

3204 So accordingly, the following effects were considered as specific effects and indicators  
3205 relevant for grouping: changes in serum T3/T4, changes in serum TSH, follicular cell  
3206 hyperplasia/hypertrophy and/or increased thyroid weight and thyroid tumours and the  
3207 specific NOAEL's were established.

3208 **Relevance of the conditions**

3209 It is mandatory to investigate effects on the thyroid system in pesticide active substance  
3210 dossiers. The effects are always addressed after 90-days exposure in rodents – usually  
3211 rats – and dogs. The following endpoints are mandatory, histopathological evaluation of  
3212 the thyroid and pituitary, while estimation of hormones (T3, T4 and TSH) is optional  
3213 (case by case). Also, histological evaluation of the thyroid glands and the pituitary is  
3214 conducted in the mandatory carcinogenicity studies in rats and mice. All species are  
3215 considered relevant for humans. The duration of exposure is considered relevant for  
3216 chronic dietary exposure and non-dietary exposure.

3217 Hazard characterisation by oral exposure is considered relevant for dietary as well as  
3218 non-dietary exposure (mainly dermal). For pesticides where the inhalatory exposure is  
3219 the main route – such studies might be required for repeated dose studies. However, this  
3220 is rare.

3221



## 3222 Uncertainties

3223 As discussed above, the rat is considered a very sensitive proxy in regard to effects on  
3224 the thyroid system. Therefore, the PPR panel noted in regard to follicular tumours;  
3225 "concerning effects on the thyroid itself, prolonged enhanced secretion by the pituitary of  
3226 TSH as a response to decreased circulating thyroid hormone levels in rat studies leads to  
3227 thyroid follicular cell hypertrophy and hyperplasia, which eventually may act as a  
3228 promoting factor in the development of benign and malignant follicular cell tumours.  
3229 Although compensatory mechanisms based on feedback loops within the hypothalamic-  
3230 pituitary thyroid axis are also operative in humans from a qualitative point of view, it  
3231 appears that humans are quantitatively less susceptible to follicular cell tumor formation  
3232 resulting from thyroid hormone system imbalance than rats, based on marked  
3233 quantitative differences in kinetics of circulating thyroid hormones and in the extent of  
3234 response to changes in thyroid hormone levels (Dellarco et al., 2006)."

## 3235 Conclusion

3236 The same effects in regard to effect on the thyroid hormone system, namely statistical  
3237 significant changes in serum T3/T4 and or TSH would be assessed differently. In the  
3238 single substance evaluation such changes, although clearly treatment related, would not  
3239 be considered as adverse effects if they are not accompanied by adverse tissue  
3240 manifestations. In regard to grouping based on toxicological profile for cumulative risk  
3241 assessment, the effects are considered as relevant specific indicative effects on the  
3242 thyroid system. Thus, different NO(A)EL's could be established based on the same  
3243 dataset and therefore, in different regulatory contexts, the same effect, although being  
3244 regarded as biologically relevant in both settings, the impact on regulatory decision  
3245 making is different.

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## Annex J – Chemical Risk Assessment

### Human health risk assessment of Cadmium in food:

**Scientific Opinion of the Panel on Contaminants in the Food Chain; The EFSA Journal (2009) 980, 1-139.**

#### Assessment strategy

Cadmium (Cd) is a heavy metal found as an environmental contaminant, both through natural occurrence and from industrial and agricultural sources. Foodstuffs are the main source of cadmium exposure for the non-smoking general population. Cadmium absorption after dietary exposure in humans is relatively low (3–5 %) but cadmium is efficiently retained in the kidney and liver in the human body, with a very long biological half-life ranging from 10 to 30 years.

For the purpose of the guidance and although the scientific assessment of cadmium had a broader content, the example below focuses only on one effect.

The kidney is the critical target organ for dietary exposure to cadmium and renal damage is characterised by cadmium accumulation in convoluted proximal tubules, thereby causing cell dysfunction and damage. The earliest signs of tubular toxicity are respectively decreased tubular reabsorption (increased excretion) of low molecular weight proteins (LMWP) and increased excretion of markers of cell shedding.

Problem: Characterise critical effect for the purpose of deriving a Health based guidance value

Identification of the Agents	Identification of the Effects	Identification of the Subjects	Identification of the Conditions
Cadmium	Critical effect: Kidney damage (cell dysfunction and damage of convoluted proximal tubules)	Humans	Biomarkers (decreased tubular reabsorption (increased excretion) of low molecular weight proteins (LMWP) and increased excretion of markers of cell shedding)

#### Data collection/ Data evaluation for each dataset

The availability of quantitative human data for both toxicokinetics (TK) and toxicodynamics (TD) provides relevant data for hazard identification and characterisation without the need to use animal data.

#### Data Evaluation (Biological Relevance)

Cadmium is bio-accumulating due to very slow renal excretion (TK), leading to excretion of biomarkers of kidney damage (TD).

#### Biological Relevance of biomarker of proximal tubular dysfunction

##### Is the effect in itself a(n) adverse/positive effect?

The CONTAM Panel based its assessment on the use of the low molecular weight protein (LMWP) beta-2-microglobuline (B2M) in urine as biomarker of Cd-induced tubular toxicity. Increased excretion of B2M is not per se associated with any objective symptom or disease. Outcome: B2M is not in itself an adverse effect.

### 3295 Is the effect essentially linked to a(n) adverse/beneficial outcome?

3296 The urinary excretion of LMWPs and the activity of some enzymes (mainly N-acetyl-  
3297 betaglucosaminidase (NAG)) in urine have been respectively used to assess tubular  
3298 dysfunction and cell damage; Urinary beta-2-microglobulin (B2M) has been widely used  
3299 as an indicator. Outcome: B2M is essentially linked to an adverse outcome

### 3300 Relevant size of the effect?

3301 In occupational exposed subjects, adverse effects of cadmium on the kidney were  
3302 observed at urinary levels of cadmium ranging from 1.1 to 15 µg/g creatinine; abnormal  
3303 levels of B2M were found in the urine of workers with urinary cadmium levels greater  
3304 than 1.5 µg/g creatinine.

3305 Based on studies on the clinical relevance of urinary B2M excretion, tubular damage and  
3306 renal function and damage the Contam Panel chose cut-off levels associated with renal  
3307 protection and irreversible kidney damage (see page 71, EFSA Journal (2009) 980, 71-  
3308 139). As an indication of abnormality, a value of 1000 µg B2M /g creatinine was set as a  
3309 high-level criterion. B2M excretion levels above this limit are likely to be irreversible  
3310 kidney damage.

3311 As a lower and more protective cut-off level, a value of 300 µg B2M /g creatinine was  
3312 chosen. Exceeding the biological cut-off of *300 µg/g creatinine* for B2M has been  
3313 associated with an accelerated decline of renal function associated with aging together  
3314 with increased mortality.

3315 Statistically-based cut-off criteria corresponding to the 95th percentile of the B2M  
3316 distribution at background urinary cadmium concentrations were also calculated. The  
3317 statistically-based cut-offs for the whole populations and for subjects over 50 years were  
3318 211 and 374 µg B2M/g creatinine, respectively.

3319 BMDs and BMDLs at various cut-offs leading to extra risks of 5 % in the total population,  
3320 and non-occupationally exposed subjects above 50 years of age were calculated.

### 3321 Calculations of BMDs and BMDLs

	Statistical cut-off <sup>a)</sup> for U- beta-2-microglobulin (µg/g creatinine)		U-beta-2-microglobulin >300 µg/g creatinine		U-beta-2-microglobulin >1000 µg/g creatinine	
	BMD5	BMDL5	BMD5	BMDL5	BMD5	BMDL5
U-Cd (µg/g creatinine) from the whole population	3.98	3.62	4.65	3.84	6.80	5.95
U-Cd (µg/g creatinine) from non-occupationally exposed subjects over 50 years	5.28	4.89	5.25	4.45	6.33	5.46

<sup>a)</sup> 211 and 374 for whole and subjects over 50 years, respectively.

3322

### 3323 Relevance of the conditions

3324 The relevance of the different BMDL5 calculated for risk assessment of the whole  
3325 population were evaluated by the CONTAM Panel

3326 Taking into account the slightly higher values for the subjects over 50 years and the  
3327 range of the BMDL5 results for the statistical and the biological cut-off limit of 300 µg  
3328 B2M / g creatinine, the CONTAM Panel selected an overall group-based BMDL5 of 4 µg  
3329 cadmium / g creatinine.

3330 The use of 300 µg B2M / g creatinine as critical effect of cadmium exposure to base the  
3331 risk assessment leads to a possible overestimation of the risk, but it allows protecting the  
3332 most sensitive groups of the population.

To account for inter-individual variations in cadmium concentration within groups, not explicitly accounted for in the BMD modelling (i.e. when calculating the lower one-sided 95 %-confidence bound for an extra risk of 5 % of producing a specified change in the urinary level of the B2M, denoted BMDL5), the CONTAM Panel modified the BMDL5 value using a chemical specific adjustment factor (CSAF) for cadmium based on the estimated variance of within group cadmium concentration. After adjustment, the CONTAM Panel identified a critical cadmium concentration of 1 µg cadmium/g creatinine in urine as a modified reference point (RP) on which to base a health based guidance value (HBGV) of cadmium dietary intake.

#### **Converting the RP to an intake value and derive a HBGV for Cd**

Subsequently, a one-compartment population toxicokinetic (TK) model was fitted to 680 paired data of cadmium intake and urinary cadmium concentrations from the Swedish Mammography Cohort study (Amzal et al., 2009). This TK model showed that a dietary intake of no greater than about 2.5 µg/kg b.w. cadmium per week would prevent 95 % of the Caucasian population from being above the modified RP of 1 µg cadmium/g creatinine in urine after 50 years of exposure (EFSA, 2009a). In order to remain below this modified RP it was calculated that the average daily dietary cadmium intake should not exceed 0.36 µg/kg b.w., and this daily intake was used to derive the TWI of 2.5 µg/kg b.w.

#### **References**

Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on cadmium in food. The EFSA Journal (2009) 980, 1–139.

## Annex K – Environmental Risk Assessment

In environmental risk assessment the protection goal is normally based on protecting populations. In some cases, it is also based on individuals, for instance for all vertebrates. Sometimes the protection goal is a function, for instance nitrification, and sometimes it includes even behaviour e.g. for bees and vertebrates.

For example, in the Avian Reproductive Test (OECD 206) the following endpoints must be assessed:

- Frequency, duration and description of signs of toxicity, along with severity, numbers affected and any remissions
- **Food consumption** and **body weight** for adults and juveniles
- Details of gross pathological examinations
- Results of residue analysis (if performed)
- **Egg production** – number of eggs laid per hen (10 weeks)
- Percentage of **cracked eggs**
- **Viability** (per cent viable embryos of eggs set)
- **Hatchability** (per cent hatching of eggs set)
- **Percentage of hatchlings** that survive to 14 days
- Number of **14 day old survivors** per hen
- **Eggshell thickness** (mm)

The test should be carried out with a minimum of three dietary concentrations of the test substance. The concentrations to be used should be based upon the results of a dietary LC50 test (OECD 205). The highest concentration should approximately be one half of the LC10. Lower concentrations should be geometrically spaced at fractions of the highest dose (e.g. 1/6 and 1/36 of the highest dose).

As a consequence of this design the power for each endpoint is different. For some endpoints the power will be very weak and some others strong and they will vary between compounds and over time.

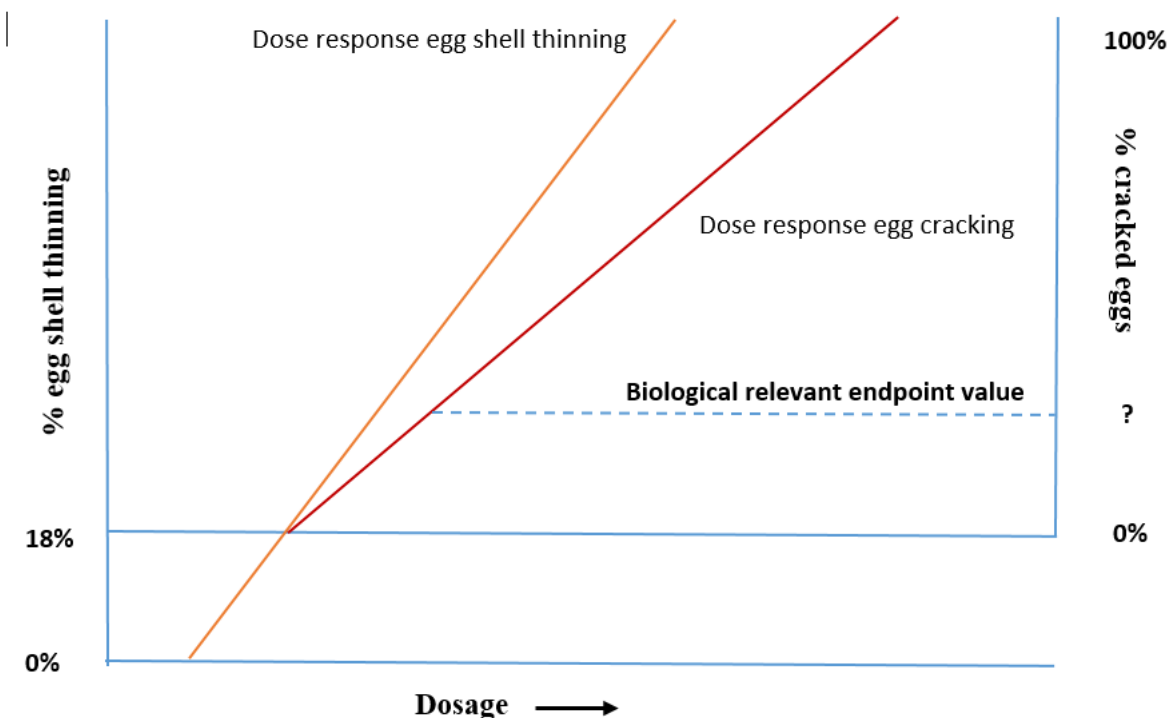
All endpoints are in principle assumed to be relevant when populations are the protection goal. The hazard assessment of the bold printed endpoints above is based on a NOEC, i.e. the highest tested concentration in which the values for the observed effect are not significantly different from the control. Of all other observations the risk assessor has to consider whether these effects could influence the survival of the population.

Although an endpoint is statistically significantly different from the control it may not be biologically relevant. In order to determine the biological relevance of an effect it should be considered whether the effect could lead to a functional deficit later on in the study, e.g. if a reduction in the weight of pups at birth leads to a decrease in level of survival. If not, then the effect may not be biologically relevant, however if there is a carry-over of effects into the number of survivors, it can be considered biologically relevant.

### Example involving egg shell thickness and cracked eggs

As stated above not all outcomes of the test are biological relevant, for instance if the LOEC (lowest observed effect concentrations) for egg shell thinning is 3% then it is generally believed that the NOEC does not have a biological relevance. It is believed that the biological relevant percentage of egg shell thinning starts with 18% (Blus 2003, EFSA

2009). The BMD dose equivalent to 18% effect can be calculated with an appropriate method. This BMD can be considered as the “NOEC” for cracked eggs (see figure below).



**Figure 5: Relation between egg shell thickness (orange line) and cracked eggs (red line). The dashed line is the line for effecting the reproduction of a bird species (e.g. when is the number of cracked eggs too much for maintaining a stable population).**

In many cases it will be difficult to point out what the biological relevant threshold of an endpoint will be. A tool that can be used is to run legislative acceptable models and to assess at which percentage a population will not be able to recover any more or when a population suffers to an unacceptable degree and to include in this assessment the uncertainty around the outcome.

A “NOEC” for cracked eggs is in view of a precautionary principle a good starting point for the risk assessment but it is probably not the value at which a population will start to show signs of decreased ability to survive.

Another approach is to include recovery in the risk assessment, which is for instance an option in aquatic risk assessment (not fish or amphibians) and terrestrial risk assessment for invertebrates. In the aquatic ecosystem a compound can be allowed on the market when recovery is seen within a period of 8 weeks but only when all important organism groups are included in the mesocosm experiment (see opinion on recovery, EFSA 2013 and 2016).

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## Glossary [and/or] abbreviations (To be completed in the final document)

Toxicokinetics (TK): Describes the processes leading to the internal concentrations of a chemical or its metabolites(s) through knowledge of absorption (A), distribution (D), metabolism (M) and excretion E (ADME).

Toxicodynamics (TD): Describes the processes that lead to the toxic effects of a chemical or its metabolites(s) once it has reached the organ(s) or tissue(s).

Reliability: Refers to the degree to which an assessment tool produces stable and consistent results. (<https://www.uni.edu/chfasoa/reliabilityandvalidity.htm>).

Protection goals: Natural resources (e.g. arthropod natural enemies, bees) or natural resource services (e.g. regulation of arthropod pest populations, pollination) that are to be protected as set out by EU.

Confounding (Taken from Wikipedia, <https://en.wikipedia.org/wiki/Confounding>)

In statistics, a confounding variable (also confounding factor, a confound, a lurking variable or a confounder) is an extraneous variable in a statistical model that correlates (directly or inversely) with both the dependent variable and the independent variable, in a way that "explains away" some or all of the correlation between these two variables.

Bradford Hill criteria (Taken from Wikipedia, [https://en.wikipedia.org/wiki/Bradford\\_Hill\\_criteria](https://en.wikipedia.org/wiki/Bradford_Hill_criteria))

(Hill, Austin Bradford.1965. The Environment and Disease: Association or Causation? Proceedings of the Royal Society of Medicine. 58; 5: 295–300.)

The Bradford Hill criteria, otherwise known as Hill's criteria for causation, are a group of guidelines that can be useful for providing evidence of a causal relationship between a putative cause and an effect, established by the English epidemiologist Sir Austin Bradford Hill (1897–1991) in 1965.

The list of the criteria is as follows:

1.Strength (effect size): A small association does not mean that there is not a causal effect, though the larger the association, the more likely that it is causal.[1]

2.Consistency (reproducibility): Consistent findings observed by different persons in different places with different samples strengthens the likelihood of an effect.[1]

3.Specificity: Causation is likely if there is a very specific population at a specific site and disease with no other likely explanation. The more specific an association between a factor and an effect is, the bigger the probability of a causal relationship.[1]

4.Temporalty: The effect has to occur after the cause (and if there is an expected delay between the cause and expected effect, then the effect must occur after that delay).[1]

5.Biological gradient: Greater exposure should generally lead to greater incidence of the effect. However, in some cases, the mere presence of the factor can trigger the effect. In other cases, an inverse proportion is observed: greater exposure leads to lower incidence.[1]

6.Plausibility: A plausible mechanism between cause and effect is helpful (but Hill noted that knowledge of the mechanism is limited by current knowledge).[1]

7.Coherence: Coherence between epidemiological and laboratory findings increases the likelihood of an effect. However, Hill noted that "... lack of such [laboratory] evidence cannot nullify the epidemiological effect on associations".[1]

- 3496 8.Experiment: "Occasionally it is possible to appeal to experimental evidence".[1]  
3497 9.Analogy: The effect of similar factors may be considered.[1]

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