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1 Guidance on Biological Relevance

2 EFSA Scientific Committee,
3 Jan Alexander, Jean-Louis Bresson, John Griffin, Susanne Hougaard, Robert
4 Luttik, Antoine Messean, André Penninks, Giuseppe Ru, Josef Rudolf Schlatter,
5 Jan Arend Stegeman, Wopke van der Werf, Henk van Loveren, Johannes
6 Westendorf, Rudolf Antonius Woutersen, Fulvio Barizzone, Bernard Bottex,
7 Nikolaos Georgiadis, Anna Lanzoni

8 Abstract

9 (Max. 300 words, no paragraph breaks; no tables, footnotes, graphs or figures. Note that
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19 **Correspondence:** xxx@efsa.europa.eu

Panel [or Scientific Committee] members: [add names in the format Name Surname, Name Surname and Name Surname].

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1 Summary

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

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

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

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

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

- 20 o Development of the assessment strategy, in this context, specification of
21 agents, effects, subjects and conditions.
- 22 o Collection and extraction of data, i.e. identification of potentially biologically
23 relevant evidence/data as specified in the Assessment strategy
- 24 o Appraisal of the relevance of the agents, subjects, effects and conditions, i.e.
25 reviewing dimensions of biological relevance for each data set.
 - 26 ▪ The agent; it should be considered whether the assessment is based on
27 the agent of concern or on a surrogate agent.
 - 28 ▪ The subject; in case proxies are used consider the relevance of effects
29 occurring in these for the subject under assessment.
 - 30 ▪ The effect; a wide variety of effects may be considered. Consideration
31 should be given as to whether the effect is causally related to exposure
32 to the agent, and the nature of the effect should also be taken into
33 account, i.a. homeostatic response, adaptive, directly or indirectly
34 adverse or beneficial. Finally for effects where the size of the effect is
35 critical, it should be assessed whether the magnitude of the effect is
36 sufficient to be of biological relevance and thereby of importance for the
37 assessment outcome. It should be noted that the biological relevance of
38 an effect can vary according to the assessment question.
 - 39 ▪ The conditions; it should be considered whether the conditions of a
40 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.

DRAFT

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1. Introduction

96

1.1. Background and Terms of Reference as provided by EFSA

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

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

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

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

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

127 In its opinion on biological relevance versus statistical significance, the EFSA
128 Scientific Committee gave a wider definition of biological relevance than just a
129 modification of a physiological system, making it more applicable to the various
130 EFSA working areas. In that opinion, biological relevant effect is defined as an
131 effect considered by expert judgement as important and meaningful enough for
132 human, animal, plant or environmental health. It implies a change that may alter
133 how decisions for a specific problem are taken (EFSA Scientific Committee,
134 2011).

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

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

144 Terms of reference

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

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

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

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

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

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

170 **1.2. Interpretation of the Terms of Reference**

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

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

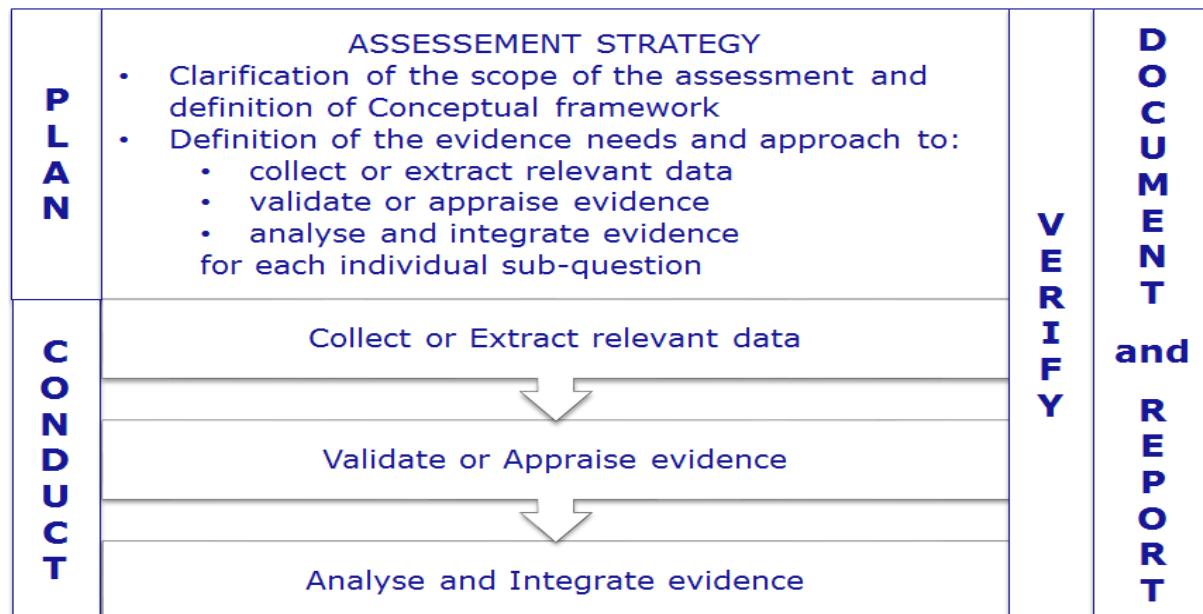
180 The purpose of this document is to discuss and provide guidance across
181 Panels/Units of EFSA on the above mentioned issues and how they should be
182 addressed during the risk assessment process. Although these issues are
183 expected to be considered consistently by the different Panels/Units,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

185

186 1.3. Relation to other relevant EFSA guidance documents

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

191



192

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

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

198 'Open EFSA' aspires both to improve the overall quality of the available
 199 information and data used for its scientific outputs and to comply with normative
 200 and societal expectations of openness and transparency (EFSA, 2009, EFSA
 201 2014). In line with this, EFSA is publishing three separate but closely related
 202 guidance documents to guide its expert Panels for use in their scientific
 203 assessments (EFSA, 2015a). These documents address three key elements of the
 204 scientific assessment: the analyses of Uncertainty, Weight of Evidence and
 205 Biological Relevance.

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

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

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

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

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

239 **1.4. Audience and degree of obligation**

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

247

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

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

254

255

256

257

258 **2. Data and methodologies**

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

263

- 264 • the problem formulation;
- 265 • the conceptual framework;
- 266 • the definition of the evidence needs; and
- 267 • the approach for:
 - 268 – Collecting or extracting relevant data;
 - 269 – Validating or appraising evidence;
 - 270 – Analysing and integrating evidence.

272

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

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

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

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

306

307 **3. Assessment**

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

311

- 312 • Development of the assessment strategy
- 313 • Collection and extraction of data
- 314 • Appraisal of the evidence.

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

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

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

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

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

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

339

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

344

349 3.1. Concepts about biological relevance

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

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

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

367 3.1.1. About responses of a biological system to exposure

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

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

379 An adverse effect might be primary (directly induced by the agent) or secondary
380 (e.g. related to stress or nutritional imbalance induced by the agent) (Lewis,
381 2002). It is important to distinguish this for the interpretation of the effect in the
382 context of the assessment question.

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

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

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

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

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

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

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

436 3.1.2. About Mode of Action and Adverse Outcome Pathway

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

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

446 The definition of Mode of action (MoA) has evolved over time and derives from
447 earlier works by the US-EPA (US EPA, 1986, 2005) and the WHO. MoA analyses
448 have been applied to a number of case studies for non-genotoxic and genotoxic
449 chemicals (WHO, 2006a,b). The current WHO definition for MoA is 'a biologically
450 plausible sequence of key events leading to an observed effect supported by
451 robust experimental observations and mechanistic data'. MoA describes key
452 cytological and biochemical events – that is, those that are both measurable and
453 necessary to obtain the observed effect – in a logical framework (Boobis et al.,
454 2006; WHO, 2009; Meek et al., 2014). In the US, MoA has been used as a term
455 to reference a mechanistic understanding of the impact of a chemical on human
456 health and to reference other terms from epidemiology including 'disease
457 signature' and 'network perturbations'. Toxicologists would also refer to the same
458 concept using the terms 'toxicity pathway, MoA, adverse outcome pathway or
459 mechanism of action' as used by the National Research Council (NRC) report,
460 Science and Decisions: Advancing Risk Assessment (2009) (NRC, 2009) and the
461 Nextgen report of the US-EPA (US-EPA, 2013). Modified Bradford Hill criteria can
462 be used to analyse the biological plausibility of key events and the weight of the
463 related evidence. Mechanism of action is defined as the specific biochemical
464 interaction through which a substance produces an effect on a living organism or
465 in a biochemical system (WHO/IPCS EHC 240). MoA does not imply full
466 understanding of mechanism of action, which refers to a detailed molecular
467 description of individual biochemical and physiological key events leading to a
468 toxic effect (Boobis et al., 2006; WHO, 2009; EFSA, 2008)

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

480 3.1.3. About thresholds

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

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

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

492
493 The World Health Organization (WHO, 2009) defines threshold as "*Dose or*
494 *exposure concentration of an agent below which a stated effect is not observed*
495 *or expected to occur*". The WHO defines the threshold dose as "*The dose at*
496 *which an effect just begins to occur—that is, at a dose immediately below the*
497 *threshold dose, the effect will not occur, and immediately above the threshold*
498 *dose, the effect will occur. For a given chemical, there can be multiple threshold*
499 *doses, in essence one for each definable effect. For a given effect, there may be*
500 *different threshold doses in different individuals. Further, the same individual*
501 *may vary from time to time as to his or her threshold dose for any effect. For*
502 *certain chemicals and certain toxic effects, a threshold dose may not be*
503 *demonstrable. The threshold dose will fall between the experimentally*
504 *determined no-observed-(adverse-)effect level and the lowest-observed-*
505 *(adverse-)effect level, both of which have been used by different scientific*
506 *groups as a surrogate for the threshold dose in the performance of risk*
507 *assessments*".

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

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

529 3.1.4. About critical effect

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

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

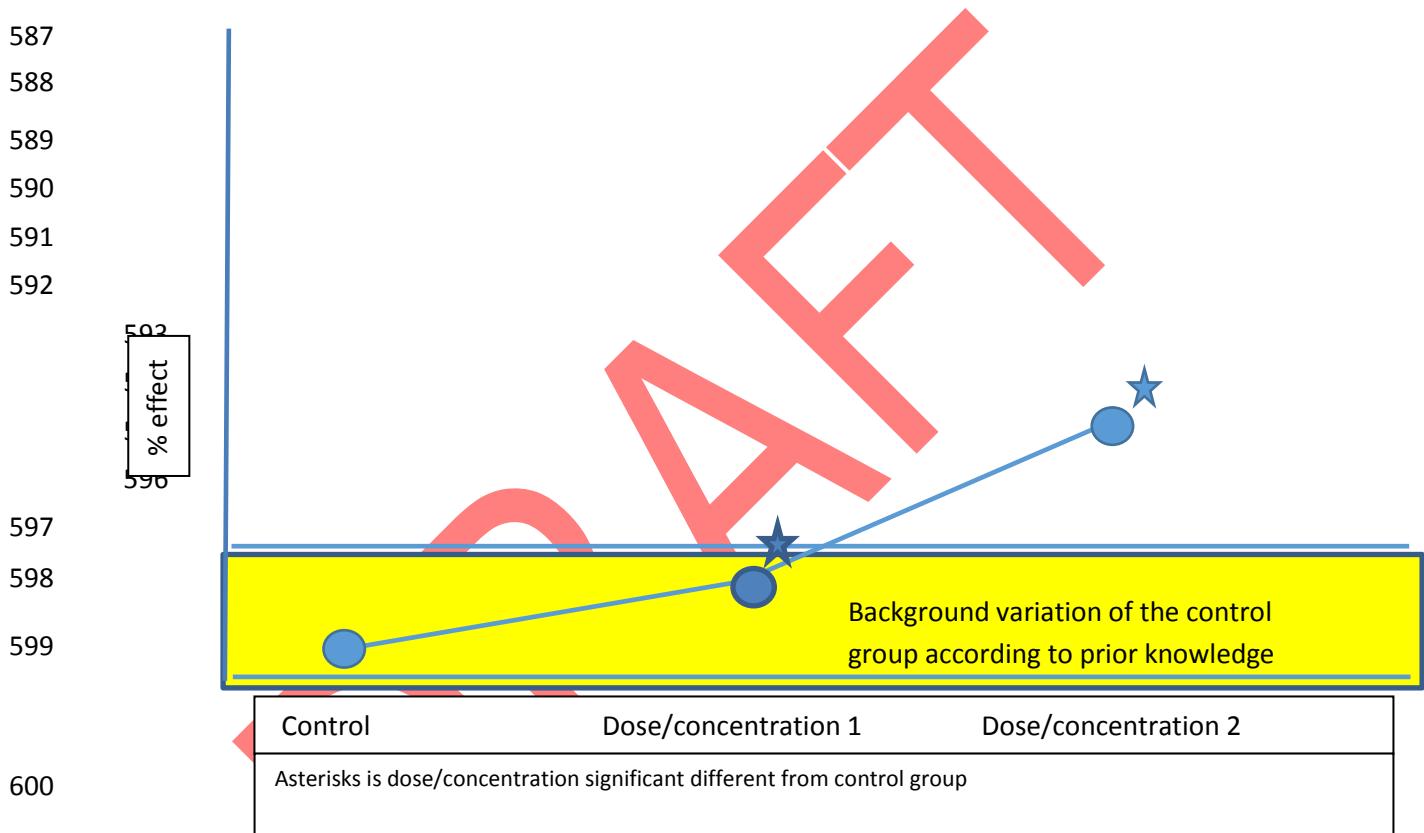
541 Assuming that temporal fluctuations in physiological parameters (e.g.
542 haematology, biochemistry) in individual healthy non-exposed animals are non-
543 adverse, the minimal magnitude of the Critical Effect Size (CES) fold change
544 above background for a number of continuous parameters of toxicity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

549 The size of an effect that would be considered biologically relevant should ideally
550 be considered before answering the assessment question. (see also EFSA, 2017,
551 chapter 2.5.2). Once this has been determined, a power analysis should be
552 carried out to determine whether a study has sufficient power of detecting the
553 defined effect as a statistically significant result, i.e. if the effect really exists.
554 "Statistically significant" does not necessarily mean "important" or "meaningful"
555 (or "biologically relevant"), as it is sometimes misinterpreted, but is a statistical
556 statement on the property and information content of the observed data (EFSA,
557 2011). In other words, a statistically significant effect may exist, but may be
558 biologically irrelevant because, although statistically significant, it is smaller than
559 the predefined biologically relevant effect size, which can be defined based on its
560 background variability. Conversely, lack of statistical significance should not be
561 the sole rationale for concluding a lack of treatment- (exposure) related effect,
562 just as statistical significance should not be the sole justification for concluding
563 on the occurrence of a treatment-related effect (OECD, 2007).

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

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

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



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

605 3.1.5. About modelling approaches

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

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

614 - to estimate reference points for hazard characterisation in toxicological
615 risk assessments by Benchmark Dose Modelling (EFSA, 2017)
616 - to assess large-scale or long-term effects on the biological system (Annex
617 K);
618 - to extrapolate the outcomes of the risk assessment to various
619 populations/receiving environments (Annex K);
620 - to assess the implications of uncertainties/assumptions on the outcomes of
621 the risk assessment, for example sensitivity analysis;
622

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

627 3.1.6. About biomarkers

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

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

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

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

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

650 3.2. Framework for consideration of 'relevance'

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

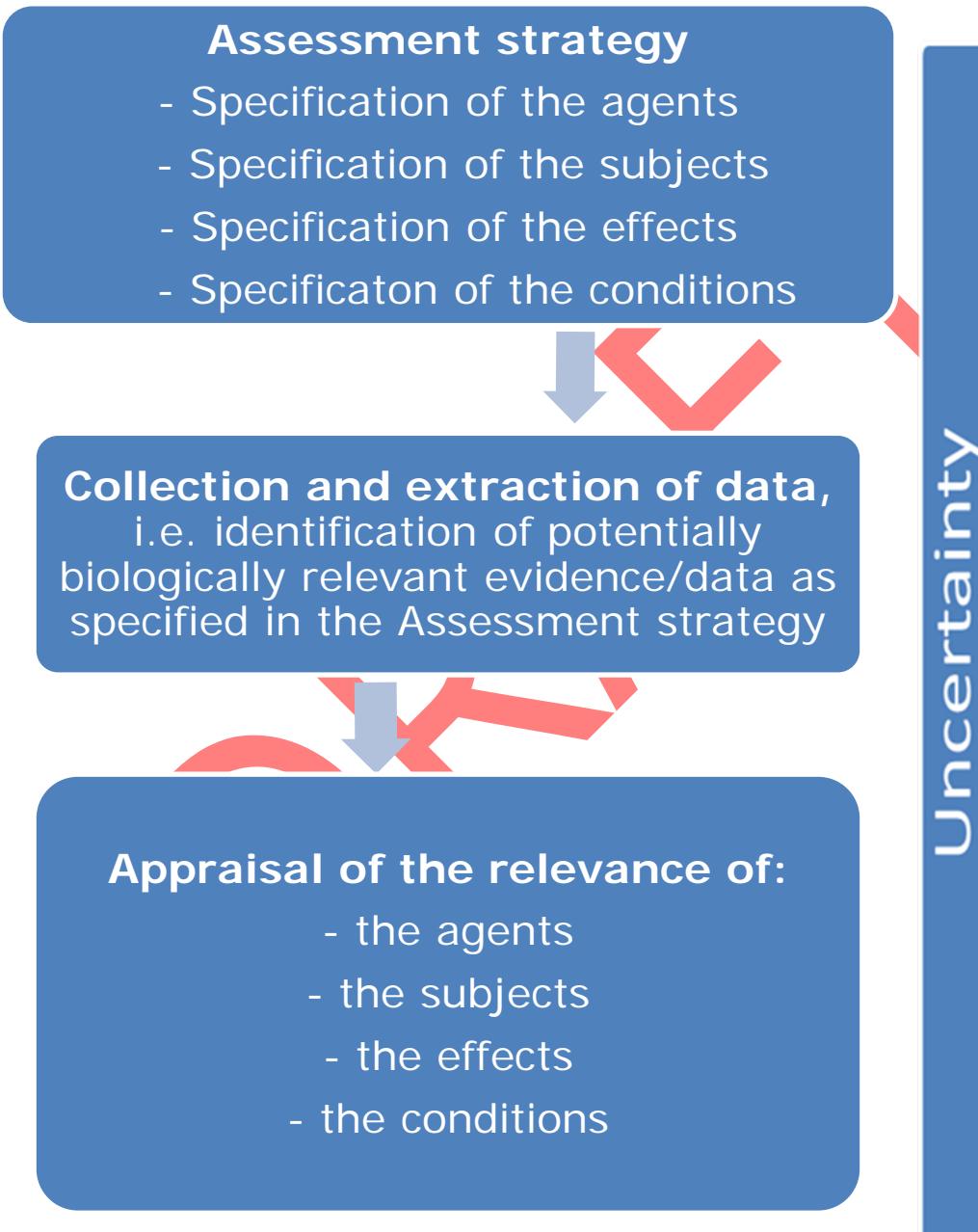
654 • Development of the assessment strategy, in this context, specification of
655 agents, effects, subjects and conditions.

656 • Collection and extraction of relevant data, i.e. identification of biologically
657 relevant evidence/data as specified in the Assessment strategy

658 • Appraisal of the relevance of the agents, subjects, effects and conditions,
659 i.e. reviewing dimensions of biological relevance for each data set.

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

662



663
664

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

667 3.2.1. Development of the Assessment strategy and relation to
668 biological relevance.

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

675

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

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

696 In standardised assessments, i.e. assessments that strictly follow guidelines or
697 guidance documents, the assessment questions are generally already defined in
698 a standard form, and there is also a standard procedure for assessing them.
699 Sometimes also regulations prescribe what kinds of data are needed for the
700 assessment. The standardised questions and procedures are part of what Codex
701 (2015) refers to as 'risk assessment policy', defined as 'Documented guidelines
702 on the choice of options and associated judgements for their application at
703 appropriate decision points in the risk assessment such that the scientific
704 integrity of the process is maintained'. This defines what questions are relevant
705 to a class of assessments, and what effects, data and analysis are relevant for
706 assessing them. It would also define what kind of biological data and effects that
707 are relevant. According to the Codex, for standardised assessments, it is
708 sufficient to confirm that the question defined by risk assessment policy is
709 relevant to the case in hand. Where it is not, the assessor needs to interpret the

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

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

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

730 3.2.2. Collection and selection of the biologically relevant data 731 according to specifications

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

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

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

¹ 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.

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

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

757 **3.2.3. Appraisal of each data set collected**

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

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

763 *a) Relevance of the agent*

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

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

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

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

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

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

792 • Is the effect dose related?

793 • Is there potential confounding? Is the response a result of confounding?

794 • Does the exposure precede the response according to a plausible time

795 scale?

796 • Is the effect biologically plausible; is there any information on the Mode of

797 Action?

798 The objective of the next step is to determine whether the observed effect, in its

799 nature and size, is relevant for the assessment question.

800 A wide range of assessment questions are considered by EFSA panels relating to

801 many different agents such as nutrient, chemicals, microorganisms, pathogens,

802 invasive species or inserted gene elements. Also the biological system assessed

803 vary widely between EFSA Panels. Hence, a large variation in the effects caused

804 by the agent can be expected. The scheme outlined below may be relevant to

805 the assessment of chemical substances, but should also be applicable to other

806 agents

807 Hence, as a first step considering biological relevance the assessor has to take

808 into account the nature of the effect caused by the agent (e.g. nutrient,

809 substance, microorganism, pathogen or invasive species) when addressing the

810 assessment question. In this context the assessor may need to determine

811 whether the effect in itself is adverse or beneficial and if not, whether it might be

812 related to such an outcome. Size or magnitude of the effect may be important

813 and is the other dimension to be considered when assessing the relevance of an

814 effect.

815 A number of questions can help to decide on the (non-)relevance of the effect

816 (see figure 4):

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

818 • Is the nature of the effect such that it is clearly adverse according to

819 the WHO definition or beneficial (see 3.1.1). For continuous data, this

820 may also be a quantitative question related to the size of the effect,

821 which then have to be considered in a next step.

822 • Does the effect represent a homeostatic response? If so, is it within the

823 homeostatic capacity of the organism or system? For continuous data,

824 this may be a quantitative question related to the size of the effect (see

825 also 3.1.1).

826 • Does the effect represent an adaptive response of a non-adverse

827 nature? An example of such a response is the caecum enlargement,

828 which is commonly seen in rodents as a result of a fibre rich diet.

829 Another example is the stimulation of the immune system following

830 exposure to microorganisms. One criterion to decide on a potential

831 adverse effect is whether or not the effect seen occurs in isolation e.g.

832 without pathological changes. (see also 3.1.1)?

833 • An example of a beneficial effect is supporting defence against

834 pathogens in the upper respiratory tract, as measured by episodes of

835 common cold and therefore biologically relevant (see Annex G). The

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

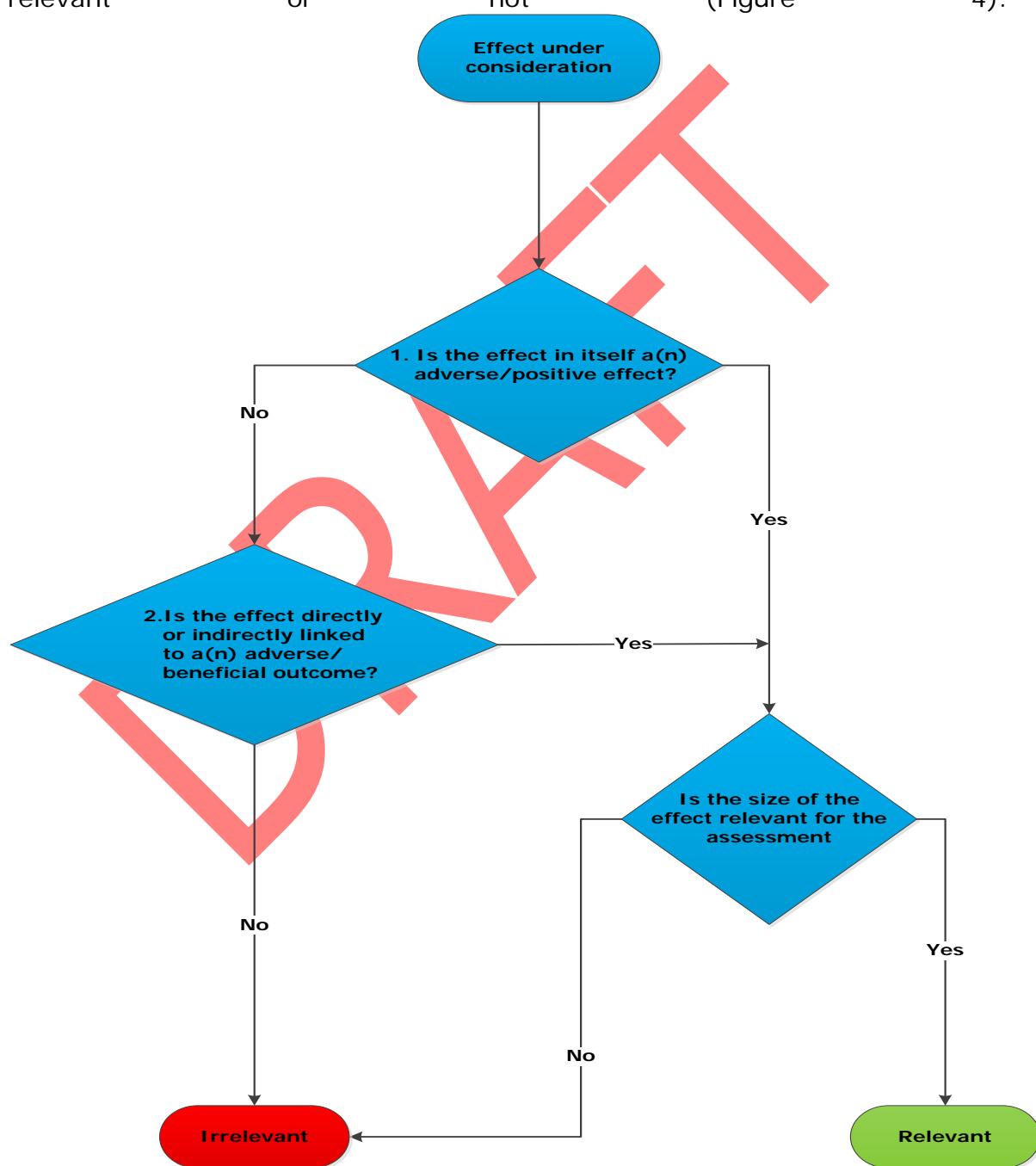
943 To assess this, similar considerations would apply as in point 2 above.

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

957 Another example is the use of biomarker to determine a population reference
958 intake for vitamin D (EFSA, 2016d). The complexity of vitamin D metabolism
959 and the unknown contribution of its endogenous synthesis do not allow
960 determining a reliable vitamin D Average Requirement in the European
961 population, hence calculating a Population Reference Intake for this
962 population. The only possible approach relies upon the use of a biomarker (of
963 status) - calcidiol or 25(OH)D - of which the serum concentration is related to
964 bone health. Indeed, there is evidence of an increased risk of adverse
965 musculoskeletal health outcomes below a certain threshold (50 nmol/L).
966 Meta-regression analysis of the relationship between 25(OH)D serum
967 concentration and total vitamin D intake allows to set an Adequate Intake of
968 15 μ g/d for the adult European population, an intake which should ensure
969 that most of the adult population will achieve a serum 25(OH)D concentration
970 near or above the target of 50 nmol/L. In this case, the relationships between
971 25(OH)D and adverse musculoskeletal health outcomes, on the one hand, and
972 total vitamin D intake, on the other hand, were considered as the biologically
973 relevant parameters.

974

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

985
986

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

988 c) *Relevance of the subject*

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

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

1000 An example of a positive effect that depends on an organ only presents in certain
1001 species is utilisation of cobalt as precursor to vitamin B12 in rumen. The bacteria
1002 present in the rumen can metabolise inorganic cobalt into vitamin B12. Mammals
1003 without a rumen as in humans, are dependent on the uptake of exogenous
1004 vitamin B12.

1005 In order to decide on the relevance of the test species to the human situation
1006 when testing chemicals, it is important also to understand the qualitative and
1007 quantitative interspecies differences, as well as the human variability in
1008 toxicokinetics (TK) and toxicodynamics (TD) processes. For a particular agent,
1009 the level of knowledge on TK and TD processes can range from very basic
1010 (external dose and toxicity) to a full quantitative understanding (external dose to
1011 internal dose to target organ dose and metabolism (TK) to specific target organ
1012 toxicity (TD) (EFSA, 2014d)

1013 In farm animals, (Annex E) beneficial effects should be demonstrated in Efficacy
1014 Studies performed with the target animals. Extrapolations can be made for other
1015 categories of the target animals (e.g. from chicken for fattening to hens for
1016 laying, or from piglets to pigs for fattening) or other species (from dairy cows to
1017 other animals used for milk production, or from chicken to other avian species).

1018 The relevance of information obtained from *in vitro* or *in silico* approaches needs
1019 to be considered in conjunction with knowledge on the MOA and other available
1020 information.

1021 In case of biological hazards; species specific pathogenecity will be considered to
1022 decide whether the effect seen in the test species is relevant for the target
1023 species.

1024 In a study by Cassard et al. (2014), animal models were used to evaluate the
1025 zoonotic potential of classical scrapie (See Annex C). Transgenic mice over-
1026 expressing the human PrP gene and homozygous and heterozygous for
1027 methionine and valine at codon 129 were inoculated intracerebrally. The Biohaz
1028 Panel concluded that the mouse lines were well established and have been shown
1029 to be susceptible to different CJD and BSE strains. Although over-expression of
1030 PrP is not a natural condition in humans, and it might have impact on some
1031 biological parameters, this can be considered a scientifically appropriate
1032 approach to modelling the molecular barrier for transmission of scrapie in
1033 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.

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

1080 outcome of the toxicity test (this can be both direction: a less toxic
1081 outcome or a more toxic outcome). It is also worthwhile to assess the
1082 vehicle used to apply the toxic compound to the test organism or the test
1083 system (often the vehicle is tested on its own).

1084 Field studies (ref to the gd document for aquatic toxicity)

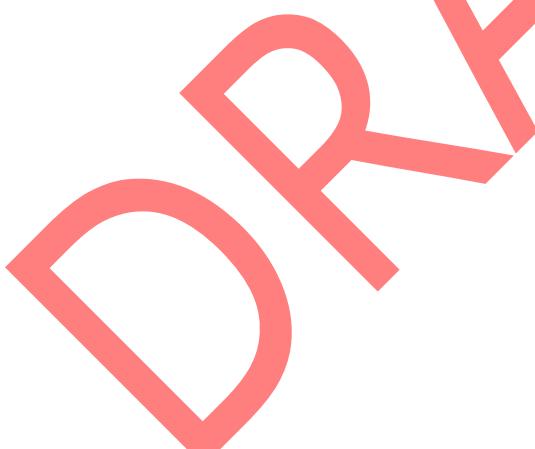
1085 • For field studies additional criteria have to be checked, for instance,
1086 whether the important animal groups are represented in the field study.
1087 These types of criteria will not be discussed in this document, but are
1088 important issues to be considered when judging whether the outcome of a
1089 test can be used in risk assessment (see for instance the guidance
1090 document for aquatic organisms (EFSA 2013)

1091 Other parameters, such as the number of animals per dose groups, number of
1092 doses tested, etc. are more related to reliability of the evidence (see the SC
1093 guidance on weight of evidence which is under development).

1094 **3.2.4. Uncertainty related to the relevance**

1095 Including evidence with less biological relevance adds to the overall uncertainty.
1096 Uncertainties arising when assessing biological relevance should be addressed
1097 and described together with other uncertainties at all stages of the assessment.
1098 General guidance on methods for assessing sources of uncertainty and their
1099 impact on assessment conclusions is provided by EFSA, 2016b, and can be
1100 applied to uncertainty arising from considering evidence that have limitations in
1101 their relevance as well as from other sources.

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1114 4. Reporting the assessment of biological relevance

1115 Assessing biological relevance should be addressed and described as part of the
1116 weight of evidence assessment. General guidance on methods for reporting
1117 weight of evidence assessment conclusions is provided by EFSA (under
1118 development), and can be applied to the assessment of biological relevance.

1119 If the assessment of the biological relevance has been conducted following a
1120 standardised procedure previously established for use in this area of EFSA's
1121 work, the assessment of the biological relevance may be reported in the manner
1122 that is normal for that standardised procedure, provided this is transparent. The
1123 standardised procedure should be referenced and its applicability to the case in
1124 hand should be explained if it is not self-evident.

1125 All other assessments of the biological relevance should be reported following the
1126 proposed framework according to the three basic steps of assessment of the
1127 biological relevance: (1) Development of the assessment strategy, including
1128 specification of agents, effects, subjects and conditions; (2) Collection and
1129 extraction of data, i.e. identification of potentially biologically relevant
1130 evidence/data as specified in the assessment strategy; (3) Appraisal of the
1131 relevance of the agents, subjects, effects and conditions, i.e. reviewing
1132 dimensions of biological relevance for each data set. This reporting should be
1133 included in the report of the weight of evidence assessment.

1134 Reporting should be consistent with EFSA's general principles regarding
1135 transparency (EFSA 2006, 2009) and reporting (EFSA 2014a, 2015). The
1136 assessment of the biological relevance should include justifying the choice of
1137 methods used, documenting all steps of the procedure in sufficient detail for
1138 them to be repeated, and making clear where and how expert judgement has
1139 been used. Where the assessment used methods that are already described in
1140 other documents, it is sufficient to refer to those. Reporting should also include
1141 referencing and, if appropriate, listing or summarising all evidence considered,
1142 identifying any evidence that was excluded; detailed reporting of the
1143 conclusions; and sufficient information on intermediate results for readers to
1144 understand how the conclusions were reached.

1145 Assessment of the biological relevance is part of the wider process of scientific
1146 assessment. Guidance on reporting other parts of the wider procedure, including
1147 evidence review, problem formulation and uncertainty analysis, is provided
1148 elsewhere (e.g. EFSA 2014b, EFSA 2015a, 2016b).

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1152 5. Conclusions and recommendations

- 1153 • This guidance document is intended to guide EFSA panels and staff in the
1154 assessment of the biological relevance of scientific evidence. When
1155 addressing the mandate, the Scientific Committee acknowledged that the
1156 issue of biological relevance in risk assessment has a broader meaning
1157 than the biological relevance of an effect as described in the Terms of
1158 Reference. In fact, it encompasses also aspects related to the definition of
1159 the problem formulation. This, in turn, guides the development of the
1160 assessment strategy, which includes the decision on which data to use for
1161 the assessment (relevance of the data).
- 1162 • Relevance is a fundamental concept in dealing with evidence and has
1163 different implications in terms of elements to be considered at different
1164 stages of the assessment and it can only be determined when the
1165 assessment question is well defined, which forms the basis for developing
1166 an assessment strategy.
- 1167 • A framework was developed in which biological relevance is considered at
1168 three main stages related to the process of dealing with evidence:
 - 1169 o Development of the assessment strategy, in this context,
1170 specification of agents, effects, subjects and conditions.
 - 1171 o Collection and extraction of data, i.e. identification of potentially
1172 biologically relevant evidence/data as specified in the Assessment
1173 strategy
 - 1174 o Appraisal of the relevance of the agents, subjects, effects and
1175 conditions, i.e. reviewing dimensions of biological relevance for each
1176 data set.
 - 1177 ▪ the agent; it should be considered whether the assessment is
1178 based on the agent of concern or on a surrogate agent.
 - 1179 ▪ the subject; in case proxies are used consider the relevance
1180 of effects occurring in these for the subject under
1181 assessment.
 - 1182 ▪ the effect; a wide variety of effects may be considered.
1183 Consideration should be given as to whether the effect is
1184 causally related to exposure to the agent, and the nature of
1185 the effect should also be taken into account, i.a. adaptive,
1186 directly or indirectly adverse or beneficial. Finally, it should be
1187 assessed whether the magnitude of the effect is sufficient to
1188 be of biological relevance and thereby of importance for the
1189 assessment outcome. It should be noted that the biological
1190 relevance of an effect can vary according to the assessment
1191 question.
 - 1192 ▪ the conditions; it should be considered whether the conditions
1193 of a test system, e.g. exposures, models, are relevant for
1194 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.

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1353 **Examples of biological relevance considerations in panel-specific**
 1354 **scientific assessments**

1355 **Annex A – AHAW**

1356 **Case study of biological relevance in the area of animal health – Risk of**
 1357 **introduction and establishment of Rift Valley Fever in the countries**
 1358 **neighbouring the EU**

1359 **Assessment strategy**

1360 Rift Valley Fever (RVF) virus is a vector borne virus that affects ruminants. Humans can
 1361 contract the infection when in close contact with infected ruminants. The causative agent
 1362 is a Bunyavirus that is endemically present in Sub-Saharan Africa. The European
 1363 Commission wanted to know whether the virus is moving its territory in Northern
 1364 direction.

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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	

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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

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1368 *Problem formulation:* What is the risk of entry and establishment of RVF into the
 1369 Mediterranean countries neighbouring the EU.

1370 **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 originated from literature review and OIE outbreak information; in general data availability was limited and available data was fragmented)

1373 - Data of movement of animals from source countries to region of concern (this is undocumented trade, so no data available; estimates were made using formal expert elicitation (Sheffield method))

1374 - Data on movements of competent vectors from source countries to region of concern (data were derived from literature and expert elicitation)

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

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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 literature, further extrapolated based on suitability of the habitats)

1386 - Geographical density of ruminants (data derived from FAO)

1387 - Temperature in the geographical regions (based on temperature records of the region)

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1391 **Data evaluation**

1392 **Relevance of the agent**

1393 Rift Valley Fever virus is a zoonotic virus of the family Bunyaviridae that has ruminant species as reservoir hosts. Infected animals suffer from fever, young animals may die and pregnant animals may abort. The infection is primarily transmitted between animals through mosquitos. Humans can contract the infection when in close contact to animals.

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1399 **Relevance of the subject**

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

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1403 **Relevance of the effect**

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

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1407 **Relevance of the conditions**

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

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1417 the influx of new susceptible animals might be insufficient for maintenance of infection)
 1418 and climate (if temperatures drop in the winter to values that do not enable the vector
 1419 cycle, infection will fade out in winter).

1420 **Overall conclusion**

1421 The assessment revealed that the introduction of RVF in the region of concern is highly
 1422 likely, but most often takes place in regions where the combined ruminant and vector
 1423 density is insufficient to result in establishment. However, according to the assessment
 1424 the region of concern has regions where RVF could become endemic.

1425 **Uncertainty**

1426 The main potential sources of uncertainties in this setting may be summarised as follows:

- 1427 • Mathematical models are a simplification of reality;
- 1428 • Uncertainty around model parameters in particular :
 - 1429 o Movement of animals from Sub Saharan to Northern Africa was derived using
 1430 expert elicitation;
 - 1431 o Vector density was mostly based on presence of suitable vector habitat only;
 - 1432 o Uncertainty regarding vector competence of vectors present in Northern Africa
 1433 o

1434 The uncertainty around the number of introductions was high (but also the lower limit of
 1435 the estimate indicates a likely introduction). Due to sparse data the uncertainty around
 1436 the vector densities is also high and it is uncertain how the competence of vectors can
 1437 vary within a certain vector species.

1438 **Case study of biological relevance in the area of animal welfare – Gas stunning 1439 and unconsciousness at slaughter**

1440 **Assessment strategy**

1441 In the slaughter process animals are killed by exsanguination. However, in order to avoid
 1442 pain and suffering, they should be rendered unconscious prior to exsanguination and
 1443 remain so until death occurs through loss of blood. In most cases, poultry are stunned
 1444 using an electric current, but recently gas stunning has gained interest due to animal
 1445 welfare advantages. It is to be expected that the industry will continue to develop new
 1446 stunning methods or modify electrical or gas stunning parameters. It is therefore
 1447 important to ensure that the new or modified stunning methods meet animal welfare
 1448 standards. Thus, an assessment protocol has been developed to evaluate new or
 1449 modified stunning methods.

Agent	Effect	Subject	Condition
Stunning method	Loss of consciousness	Poultry	Slaughter

1450

1451 *Problem formulation:* To maintain good standards of animal welfare, it is important to
 1452 establish whether the new or modified stunning method (a) produces immediate loss of
 1453 consciousness, (b) if loss of consciousness is not immediate, does it cause avoidable pain
 1454 and suffering during the induction of unconsciousness, and (c) is the duration of
 1455 unconsciousness long enough to avoid recovery of consciousness either prior to slaughter
 1456 or during exsanguination.

1457 **Evidence/data needed to address the question**

1458 Firstly, the brain mechanism associated with the induction of unconsciousness by a new
1459 or modified stunning method needs to be clearly explained. The state of consciousness
1460 can be ascertained under the controlled laboratory conditions by recording spontaneous
1461 as well as evoked activity in the brain using electroencephalograms (EEGs) before and
1462 after the application of a stunning method. The unique brain states that are incompatible
1463 with persistence of consciousness should be demonstrated using EEGs. Secondly, the
1464 correlation between EEG evidence and animal based indicators (as proxies) of
1465 unconsciousness for monitoring in slaughterhouses also need to be established. Thirdly,
1466 the duration of unconsciousness should be determined. In essence, the duration of
1467 unconsciousness should be longer than the sum time interval between the end of
1468 stunning and cutting blood vessels in the neck and the time it takes for the onset of
1469 death through exsanguination. Finally, the maximum permissible time between the end
1470 of stunning and neck cutting should be established.

1471 **Data evaluation**

1472 **Relevance of the agent**

1473 Killing animals by exsanguination is a potentially painful process and the sources of pain
1474 includes, (a) cutting soft tissues, nerves and blood vessels in the neck (sawing motion or
1475 making several cuts), (b) direct activation of neurones by the blade as it transects the
1476 nerves produce intense pain and (c) the sensations produced during the injury discharge
1477 is likely to be an amalgam of all such inputs, and the overall effect is likely to be a sense
1478 of shock, comparable to an electric shock.

1479 **Relevance of the subject**

1480 Poultry is the relevant subject for the question, because it is also the target species for
1481 slaughter.

1482 **Relevance of the effect**

1483 The pain and suffering at exsanguination can be prevented by implementing pre-
1484 slaughter stunning of animals, i.e. rendering them unconscious prior to exsanguination.

1485 **Relevance of the conditions**

1486 The tests are done with the target species in a slaughterhouse setting. In this context,
1487 the brain of an animal is considered to be the seat of consciousness

1488 **Overall conclusion**

1489 Useful to test stunning methods according to the guideline

1490 **Uncertainty**

1491 Establishing neuronal correlates of unconsciousness remains to be a challenge. For
1492 example, the magnitude of changes occurring in the EEG considered to be incompatible
1493 with persistence of consciousness varies widely. The correlation between EEG criteria and
1494 animal based indicators of unconsciousness is not widely reported, and hence, rely on
1495 expert opinion.

1496 **References**

1497 EFSA Panel on Animal Health and Welfare (AHAW); Scientific Opinion on Rift Valley fever.
1498 EFSA Journal 2013;11(4):3180. [48 pp.] doi:10.2903/j.efsa.2013.3180.

1499 **Annex B – ANS**

1500 **Re-evaluation of aspartame (E951) as a food additive**

1501 **Introduction**

1502 Following a request from the European Commission, the Panel on Food Additives and
 1503 Nutrient Sources added to Food (ANS) of the European Food Safety Authority (EFSA) was
 1504 asked to deliver a scientific opinion on the re-evaluation of aspartame (E 951) as a food
 1505 additive.

1506 Aspartame (E 951) is a dipeptide of L-phenylalanine methyl ester and L-aspartic acid
 1507 bearing an amino group at the α -position from the carbon of the peptide bond (α -
 1508 aspartame). The major hydrolysis and degradation products of aspartame are L-
 1509 phenylalanine, aspartic acid, methanol and 5-benzyl-3,6-dioxo-2-piperazine acetic acid
 1510 (DKP).

1511 For the purpose of the guidance and although the scientific assessment of Aspartame had
 1512 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)

1513

1514 **Assessment strategy**

1515 The re-evaluation of aspartame included the assessment of the safety of its gut
 1516 hydrolysis metabolites methanol (which is oxidized to formaldehyde), phenylalanine and
 1517 aspartic acid. The hepatic enzyme phenylalanine hydroxylase (PAH) is necessary to
 1518 metabolize the amino acid phenylalanine to the amino acid tyrosine. When PAH activity is
 1519 reduced, circulating phenylalanine levels will increase. Humans heterozygous for PAH
 1520 mutations, show a slightly reduced capacity to metabolize phenylalanine compared to
 1521 normal individuals. Individuals homozygous for PAH mutations, phenylketonuria (PKU)
 1522 patients, have a markedly reduced capacity for phenylalanine metabolism. There is long
 1523 established evidence for increased severity and frequency of adverse developmental
 1524 effects with high phenylalanine plasma levels in human patients with phenylketonuria
 1525 (PKU). Maternal PKU syndrome refers to the teratogenic effects of PKU during pregnancy.
 1526 In untreated pregnancies wherein the mother has classic PKU with a plasma
 1527 phenylalanine level greater than or equivalent to 1200 μ M (20 mg/dL), abnormalities in
 1528 offspring occur at high frequencies.

1529 The pathogenesis of this syndrome is unknown; it may be related to inhibition by
 1530 phenylalanine of neutral amino acid transport across the placenta or to direct toxicity of
 1531 phenylalanine and/or a phenylalanine metabolite (phenylpyruvic acid) in certain fetal
 1532 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 15th 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

1576 **Relevance of the effect(s)**

1577 The key effects observed in the reproductive studies with rats and rabbits related to a
1578 specific life stage acknowledged to be critical in both species and to humans. A spectrum
1579 of effects was observed in the rats and rabbits, particularly maternal toxicity and growth
1580 restriction of the offspring. The latter effect was recognized as an important outcome in
1581 humans because it was associated with an increased risk of perinatal mortality and
1582 morbidity.

1583 **Relevance of the conditions**

1584 The available reproductive and developmental toxicity studies on aspartame comprised
1585 nine studies, one in mice and eight in rats. In addition, eight embryotoxicity and
1586 teratogenicity studies were performed in rabbits, four with administration of aspartame
1587 by diet and four by gavage.

1588 The best estimate of the critical effect level of phenylalanine exposure without damage to
1589 the offspring is 330 to 360 μ M. In calculating a safe level of aspartame exposure (based
1590 on plasma phenylalanine concentrations), the worst-case scenario was applied, that took
1591 into account that intake of aspartame occurs in combination with a meal leading to
1592 circulating plasma phenylalanine concentrations of 120 μ M.

1593 The concentration of plasma phenylalanine derived from aspartame was, therefore, set to
1594 240 μ M (i.e. 360 μ M minus 120 μ M).

1595 Based on **modelling**, a plasma phenylalanine concentration of 240 μ M would result from
1596 the administration of a bolus dose of 103 mg aspartame/kg bw to a normal subject.

1597 For a PKU heterozygous individual the concentration of 240 μ M would be reached by the
1598 administration of a bolus dose of 59 mg aspartame/kg bw.

1599 **Uncertainties**

1600 The following main assumptions were made based on the proposed MoA:

- 1601 • Phenylalanine plasma level of 360 μ M is the threshold for developmental effects.
- 1602 • The diet results in phenylalanine plasma level not exceeding 120 μ M.
- 1603 • Peak plasma phenylalanine concentration can be used in the dose-response
1604 modelling as surrogate of steady-state plasma phenylalanine concentration.
- 1605 • Bolus administration of aspartame can be used in the dose-concentration
1606 modelling of plasma phenylalanine to represent a more typical pattern of
1607 aspartame intake.
- 1608 • The 95th percentile confidence interval of the lower bound estimate of the
1609 aspartame dose- plasma phenylalanine concentration curve provides a safe limit
1610 for plasma phenylalanine for the entire population (with the exception of
1611 homozygous PKU patients).
- 1612 • The increase in plasma phenylalanine concentrations following aspartame
1613 administration will be the same in the general population as in individuals
1614 heterozygous for PKU.
- 1615 • Reproductive and developmental toxicity of aspartame is solely dependent on
1616 systemic exposure to phenylalanine.
- 1617 • There is no requirement for a pharmacodynamic uncertainty factor (a sensitive
1618 human population (PKU patients) was used to define the threshold).
- 1619 • There is no requirement for a pharmacokinetic uncertainty factor (the aspartame
1620 plasma phenylalanine concentration was based on a more sensitive human sub-
1621 population (PKU heterozygous)).

1622 It was not possible to place a specific numerical value on the uncertainties related to
1623 these assumptions, but the aforementioned evaluations and considerations are more
1624 likely to overestimate than underestimate any potential developmental risk. Therefore it
1625 is not illogical to conclude that the results of the uncertainty analysis further support the
1626 conclusion, that there is no safety concern for aspartame at the current ADI in normal
1627 and heterozygous subjects.

1628 **References**

1629 EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to
1630 Food), 2013. Scientific Opinion on the re-evaluation of aspartame (E 951) as a
1631 food additive. EFSA Journal 2013;11(12):3496, 263 pp.
1632 doi: 10.2903/j.efsa.2013.3496

DRAFT

1633 **Annex C – BIOHAZ**1634 **Case study of biological relevance in the area of biological hazards -**
1635 **Zoonotic potential of classical scrapie**1636 **Introduction**

1637 Transmissible spongiform encephalopathies (TSEs) are a group of progressive conditions
1638 that affect the brain and nervous system of many animals, including humans. Unlike
1639 other kinds of infectious disease, the infectious agent in TSEs is believed to be a protein,
1640 called the prion protein. Misshapen prion proteins are transmissible and are able to
1641 induce abnormal folding of specific normal cellular proteins that are found most
1642 abundantly in the brain: they carry the disease between individuals and cause
1643 deterioration of the brain. TSEs are unique diseases in that their aetiology may be
1644 genetic, sporadic, or infectious via ingestion of infected materials and via
1645 iatrogenic means (e.g., blood transfusion). Prion diseases of humans include sporadic
1646 Creutzfeldt-Jacob disease (sCJD), new variant Creutzfeldt–Jakob disease (vCJD),
1647 Gerstmann-Straussler-Scheinker syndrome, fatal familial insomnia and kuru. Prion
1648 diseases of livestock include bovine spongiform encephalopathy (BSE) in cattle, classical
1649 scrapie in sheep and chronic wasting disease in cervids.

1650 Host genetics substantially influence these diseases. In humans, familial prion diseases
1651 are closely associated with mutations in the prion protein gene, and the
1652 methionine/valine polymorphism at codon 129 appears to influence susceptibility,
1653 incubation period and in some respects disease phenotype.

1654 One of the main questions in relation to the animal TSEs is their ability to infect humans.
1655 BSE is the only TSE agent identified as zoonotic. However, it has been hypothesised that
1656 other prions associated with animals such as classical scrapie can infect humans. For
1657 disease to develop in the case of exposure through foodstuffs, there must be exposure to
1658 a sufficient dose of the agent, the agent must be taken up from the gastrointestinal tract,
1659 enter the nervous system and be successfully transported to the neuronal cell bodies in
1660 the central nervous system. The infecting agent must then be able to 'convert' the
1661 cellular prion protein (PrP^C) to the abnormal form of the prion protein (PrP^{Sc}) at a rate
1662 which enables accumulation of sufficient PrP^{Sc} to cause disease within the life-span of the
1663 host.

1664 **Assessment strategy**

1665 In a paper, 'Evidence for zoonotic potential of ovine scrapie prions', published in *Nature*,
1666 Cassard et al. (2014) studied the zoonotic potential of classical scrapie by bioassay in
1667 mice, in which a range of characteristics were assessed. These included incubation
1668 periods and neuropathological characteristics. The authors concluded that the results
1669 demonstrated that scrapie prions have zoonotic potential and raise new questions about
1670 the possible link between human and animal prions. The European Commission asked the
1671 BIOHAZ Panel to scientifically appraise the paper considering the limitations, assumptions
1672 and uncertainties associated with the study design and outputs.

1673

1674

1675

1676 In line with the framework set out in Figure 1 of the main document for consideration of
 1677 relevance, the agent, effect, subject and conditions can be considered as follows:

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

1678

1679 Hence, the problem can be formulated in these terms: in humans can the consumption of
 1680 meat from classical scrapie infected sheep result in the development of a Transmissible
 1681 Spongiform Encephalopathy?

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

1683 The evidence for the zoonotic potential of classical scrapie, as set out in the paper by
 1684 Cassard et al. (2014), was evaluated by the Biohaz Panel using expert judgement.

1685 **Appraisal of the evidence**

1686 **Relevance of the agent**

1687 A spectrum of strains is responsible for classical scrapie in sheep, and there may be
 1688 variability in properties that affect the ability to cross the species barrier. There is
 1689 experimental evidence that some isolates may not be completely stable, and their
 1690 fundamental properties may shift on transmission. There is also potential heterogeneity
 1691 of geographical distribution of individual strains.

1692 In the study by Cassard et al. (2014), six different isolates of classical scrapie were used.
 1693 These had been previously studied in other animal models and showed some degree of
 1694 biological variability. The Biohaz Panel concluded that the deliberate selection of
 1695 biologically variable scrapie isolates represents an important new aspect compared to
 1696 previous studies on the subject, given the known diversity within the group of TSE
 1697 agents identified as classical scrapie. The Panel further concluded that no case selection
 1698 will conclusively and comprehensively ever represent the total potential field exposure,
 1699 but the Cassard et al. (2014) study made a good, rational and supported choice of
 1700 isolates designed to be distinct from one another, to represent some of the possible
 1701 range of field strains.

1702 In conclusion, the isolates used in the study by Cassard et al. (2014) were considered
 1703 relevant for the problem under investigation. However, evidence derived from a limited
 1704 number of classical isolates cannot be extrapolated to represent the whole biological
 1705 variability of classical scrapie.

1706 **Relevance of the subject**

1707 In the study by Cassard et al. (2014), animal models were used to evaluate the zoonotic
 1708 potential of classical scrapie. Transgenic mice over-expressing the human PrP gene and
 1709 homozygous and heterozygous for methionine and valine at codon 129 were inoculated
 1710 intracerebrally. Historically, laboratory studies using animal models were carried out
 1711 using wild type mice. However, a substantial proportion of human and animal TSE
 1712 isolates cannot be propagated into conventional mice models, which limits the usefulness
 1713 of this system to characterize and compare TSE agents circulating in the field. More

1714 recently, transgenic mice with PrP derived from different species have been increasingly
1715 used in experimental transmission of TSE agents. Transgenic mice that are homozygous
1716 for methionine at codon 129 of the human PrP gene have been previously shown to be
1717 fully susceptible to human TSEs, such as sCJD and vCJD and, to a much lesser extent,
1718 cattle BSE, the only animal TSE with confirmed zoonotic potential identified so far.

1719 Different lines of transgenic mice that express the human PrP gene were used in the
1720 study, namely the tg340, tg361 and tg650 mouse lines. The tg340 mouse line expresses
1721 methionine approximately fourfold more than normal human brain tissue at codon 129.
1722 The tg650 mouse line overexpresses methionine sixfold at the same codon. The tg361
1723 mouse line overexpresses valine at codon129 at fourfold levels. A breeding cross
1724 between the tg340 and the tg361 provided mice that overexpress both M129 and V129
1725 alleles at similar levels. Although PrP over-expression might circumvent the low
1726 susceptibility of gene-targeted tg mice, it is worth noting that an inevitable limitation of
1727 such transgenic mice is that only one human gene is present in the model, while disease
1728 susceptibility and incubation period are inevitably multi-factorial. Additionally, if the time
1729 taken for the conversion of human PrP^c to PrP^{Sc} exceeds the lifespan of the mouse, this
1730 may give a 'false negative' outcome.

1731 The Biohaz Panel concluded that the mouse lines used by Cassard et al. (2014), in
1732 particular tg650 and tg340, are well established and have been shown to be susceptible
1733 to different CJD and BSE strains. Although over-expression of PrP is not a natural
1734 condition in humans, and it might have impact on some biological parameters, this can
1735 be considered a scientifically appropriate approach to modelling the molecular barrier for
1736 transmission of scrapie in humans despite some limitations of these transmission models,
1737 as mentioned above.

1738 **Relevance of the effect**

1739 In the study, serial passages were used in the transgenic mice for each of the six
1740 different strains of classical scrapie. Serial passages of bovine BSE, human sCJD isolates
1741 and human vCJD isolates were also carried out in these mouse lines for comparison with
1742 the classical scrapie isolates. Based on the attack rates observed after serial passages in
1743 transgenic mice, the potential for classical scrapie transmission is: i) low or absent in
1744 MM129 mice; ii) low or absent in MV129 mice; iii) absent in VV129 mice. The data
1745 suggest that BSE is still more efficient than scrapie in MM129 mice, while a single scrapie
1746 isolate would be more efficient than BSE in MV129 mice

1747 Moreover, the study also showed that the serial transmission of different scrapie isolates
1748 in humanised transgenic mice led to the propagation of prions that were phenotypically
1749 identical to those that cause sporadic Creutzfeldt Jakob Disease (sCJD) in humans.

1750 In summary, the effect shown by Cassard et al. (2014) was considered relevant. The
1751 study showed that transgenic mice could be infected with classical scrapie strains but
1752 that the transmission was less efficient than for bovine BSE. It should also be noted that
1753 while serial passage maximises the chance of detecting the propagation of TSE agents, it
1754 does not mimic natural exposure in humans.

1755 **Relevance of the conditions**

1756 Intracerebral inoculation is a widely accepted and appropriate choice of inoculation in
1757 mouse models. This method can be used to assess the permeability of the transmission
1758 barrier at the molecular level, i.e. the conformational compatibility between the infecting

1759 prion strain and the PrP^c of the recipient species. This is an important factor which limits
1760 the propagation of prion agents among different species. Successful amplification of PrP^{Sc}
1761 would indicate that the TSE strain has the potential to convert human PrP^c.
1762

1763 Natural exposure in man is believed to involve the oral route through the consumption of
1764 meat from an infected animal. In this respect, the inoculation route used in the mouse
1765 model does not represent an ideal strategy for the investigation of zoonotic potential
1766 since the involvement of the digestive system, the rest of the lymphoreticular system,
1767 the enteric nervous system and peripheral nervous system have been bypassed by the
1768 direct deposition of the prions in the brain.

1769 Therefore, it was concluded that the inoculation route used by Cassard et al. (2014)
1770 cannot reproduce field conditions and does not mimic natural exposure.

1771 **Overall conclusion**

1772 The Biohaz Panel concluded that the paper by Cassard et al. (2014), provides evidence
1773 that some classical scrapie isolates can propagate in humanised transgenic mice and
1774 produce prions that on second passage are similar to those causing one form of sporadic
1775 Creutzfeldt-Jakob disease (sCJD). However, the Biohaz Panel also concluded that the
1776 results from the study raise the possibility that scrapie prions have the potential to be
1777 zoonotic but do not provide evidence that transmission can or does take place under
1778 field conditions.

1779 **Uncertainty**

1780 The main potential sources of uncertainties in this experimental setting may be
1781 summarised as follows:

- 1782 • Evidence derived from a limited number of classical isolates cannot be extrapolated
1783 to represent the whole biological variability of classical scrapie;
- 1784 • The use of an animal model and the over-expression of PrP may not allow a direct
1785 extrapolation to human population;
- 1786 • Subsequent serial passages were thought as a way to overcome the problem of
1787 allowing a longer incubation period in mice: the occurrence of a similar condition is
1788 not realistic in the field.
- 1789 • The intracerebral inoculation route used by Cassard et al. (2014) cannot reproduce
1790 field conditions and does not mimic natural exposure.

1792 **Reference**

1793 Cassard H, Torres JM, Lacoux C, Douet JY, Benestad SL, Lantier F, Lugan S, Lantier I,
1794 Costes P, Aron N, Reine F, Herzog L, Espinosa JC, Beringue V and Andreoletti O, 2014.
1795 Evidence for zoonotic potential of ovine scrapie prions. *Nature Communications*, 5, 5821.

1796 Annex D – CEF**1797 Evaluation of the toxicity of BPA for humans considering all relevant
1798 toxicological information****1799 Assessment strategy**

1800 The EFSA scientific Panel on Food Contact Materials, Enzymes, Flavourings and
1801 Processing Aids (CEF) has re-assessed in 2015 the risks to public health from BPA
1802 exposure by evaluating the toxicity of BPA for humans, including for specific (vulnerable)
1803 groups of the population (e.g. pregnant women, infants and children, etc.) and
1804 considering all relevant toxicological information available. Exposure assessment was
1805 performed for various groups in the population and finally human health risks were
1806 characterised taking into account specific groups of the population [EFSA Journal 2015;
1807 13(1): 3978].

1808
1809 Although the scientific assessment of BPA had a broader content, for the purpose of this
1810 guidance the example below focuses only on one effect.

1811 1812 Specification of the agent

1813 Bisphenol A (BPA) is an organic chemical used as a monomer in the manufacture of
1814 polycarbonate plastics and epoxy resins and as an additive in plastics. Polycarbonates are
1815 used in food contact materials such as reusable beverage bottles, infant feeding bottles,
1816 tableware (plates and mugs) and storage containers. Epoxy resins are used in protective
1817 linings for food and beverage cans and vats.

1818 The scientific debate on the risks for public health of BPA is focussed on its endocrine-
1819 active properties, which might adversely impact physical, neurological and behavioural
1820 development. In addition, other perturbations of physiology, both in animals and
1821 humans, have been brought in relationship to the endocrine-active properties of BPA.
1822 Among these are e.g. obesity, modification of insulin-dependent regulation of plasma
1823 glucose levels, perturbation of fertility, proliferative changes in the mammary gland
1824 possibly related to the development of breast cancer, immunotoxicity and adverse effects
1825 on the cardiovascular system (for an overview see NTP-CERHR, 2007, 2008, EFSA, 2006,
1826 2008, 2010, and ANSES, 2011, 2013).

1827 Specification of the subject

1828 The question of interest resulting from this risk assessment of BPA was: 'What is the
1829 biological relevance for human health of the observed proliferative and morphological
1830 changes in the mammary gland following exposure to BPA and the possible relevance for
1831 the development of breast cancer'?

1832 Specification of the effects

1833 To update the risks to public health from BPA exposure, the complex BPA toxicity was re-
1834 evaluated by EFSA in 2015 using a Weight of Evidence (WoE) approach to identify the
1835 critical toxicological effects [EFSA Journal 2015; 13(1): 3978]. The effects on kidney
1836 weight were considered critical endpoints and taken forward to hazard characterization to
1837 assess a reference point (BMDL10) for the derivation of a health-based guidance value
1838 (TDI). As the scientific evidence for observed reproductive- and developmental-effects,
1839 neurological-, neurodevelopmental- and neuroendocrine-effects, immune effects,
1840 cardiovascular effects and metabolic effects was not sufficient, they were not taken

1841 forward for risk characterisation, but these effects were included in the uncertainty
1842 evaluation.

1843 In the WoE approach used for hazard identification, next to the general toxicity effects on
1844 kidney weight, also proliferative and morphological changes in the mammary gland were
1845 reported in several new toxicity studies and considered "likely" (likely refers to 66-100%
1846 probability), although no reference point (BMDL10) could be calculated.

1847 These proliferative responses and possibly enhanced sensitivity to mammary gland
1848 carcinogens seen in animal studies might be of relevance for human health and were
1849 therefore included in the risk assessment.

1850 **Collection of data relevant to the problem formulation**

1851 Earlier evidence for BPA effects on cell proliferation and differentiation and morphological
1852 changes potentially related to tumour induction in the mammary gland [EFSA Panel on
1853 Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), 2010] were
1854 supported by new toxicity studies published from 2010 onwards. A number of these new
1855 laboratory animal studies in rodents (rats and mice, including a.o. transgenic mouse
1856 models and DMBA mammary tumour mouse model) and a monkey study, have reported
1857 effects on mammary tissue (mammary tumour induction, enhancement of mammary
1858 tumour growth and/or proliferative changes in mammary gland) after prenatal, perinatal
1859 and adult exposure to BPA.

1860 Overall, using expert judgment the CEF Panel concluded that although there were
1861 methodological weaknesses in all these studies with the exception of a US FDA/NCTR
1862 subchronic toxicity study, which was a detailed guideline study conducted in accordance
1863 with GLP, they provide further evidence that BPA may enhance mammary epithelial
1864 proliferation in animal models.

1865 However the proliferative changes in the mammary gland reported in these new studies,
1866 including a non-human primate study, are considered insufficient to conclude that there
1867 is a link to cancer development in later life. Nevertheless, there might be a possible role
1868 of BPA in increasing the susceptibility to mammary gland carcinogenesis.

1869 **Relevance of the agent**

1870 Although the exact mode of action in respect to the reported proliferative changes in the
1871 mammary gland is not clarified they may well fit with the conclusions of the mechanistic
1872 studies in which it is shown that BPA affects a number of receptor-dependent and
1873 independent signalling pathways, resulting in effects on hormone homeostasis and gene
1874 expression as well as cytogenetic and epigenetic effects.

1875 It was concluded in the Panel that no single clearly defined mode of action of BPA can be
1876 identified that can contribute substantially to the understanding of the potential effects of
1877 BPA in humans. However, given that BPA appears to have multiple modes of action at
1878 the cellular level, and at least some of these MoAs involve cellular responses that are
1879 highly conserved across species (e.g. binding to oestrogen or androgen receptors), the
1880 relevance for humans of the variety of effects that have been reported for BPA in
1881 mechanistic studies cannot be totally discounted.

1882

1883 Relevance of the subject

1884 Although there is no convincing evidence that BPA is carcinogenic in animals when
1885 exposed to adults or during pre- and post-natal (during lactation) development, a large
1886 number of the animal studies suggest that BPA can have a proliferative/developmental
1887 advancement effect on mammary tissue, and may also have an effect on tumour growth
1888 in animal models, particularly in sensitive transgenic models or when followed by a
1889 treatment with a complete carcinogen (DMBA).

1903 In relation to possible carcinogenic effects of BPA in animals when exposed pre- and
1904 post-natally (during lactation), several studies (5) used the dimethylbenzanthracene
1905 (DMBA) mammary tumour mouse model to assess the effects of fetal or postnatal
1906 exposure to BPA on the development of mammary tumour in adults. Overall, increased
1907 susceptibility to development of mammary cancer, decreased tumour latency and
1908 increased tumour multiplicity was reported.

1909 Also studies were performed in transgenic mouse models, such as an adult knockout
1910 mouse model of mammary neoplasia, showing increased epithelial cell proliferation and
1911 hyperplasia in mammary glands of adult BRCA1* knockout mouse upon BPA exposure via
1912 osmotic pumps. In addition, in a female transgenic MMTV-erbB2/neu mice susceptible to
1913 develop mammary carcinoma, BPA-treatment via drinking water resulted in a decreased
1914 tumour latency and increased tumour multiplicity, enhanced tumour volume and higher
1915 incidence of lung metastasis.

1916 Also the US FDA/NCTR subchronic (90-day) toxicity study provided some evidence of a
1917 BPA-related effect in the mammary gland of female rats. Mammary gland duct
1918 hyperplasia of minimal severity was reported in the female groups examined at Post
1919 Natal Day (PND) 21 and in the high dose female BPA groups examined at PND 90.

1920 Taken together, as intra-ductal hyperplasia in the mammary gland is observed in humans
1921 and is considered as a precursor of ductal carcinoma both in rodents and in humans, this
1922 lesion is considered of relevance when studied in animals (e.g. rodents) to predict cancer
1923 in the human mammary gland and is considered as adverse.

1924 Relevance of the effect

1925 Intra-ductal hyperplasia in the mammary gland is observed in humans and is considered
1926 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
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2014 presence of bisphenol A (BPA) in foodstuffs: Executive summary. EFSA Journal 2015;13
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DRAFT

2046 **Annex E – FEED**

 2047 **Example from FEEDAP**

 2048 **1. Problem formulation**

2049 The scientific evaluation of feed additives by EFSA includes:

- 2050 • The safety of the additive for the target animals
- 2051 • The safety of the additive for the consumer (human health)
- 2052 • The safety of the additive for the user/worker
- 2053 • The safety of the additive for the environment
- 2054 • The efficacy of the additive

2055 The assessment of feed additives is a standardised process, which follows legal guidelines
 2056 and guidance documents. The assessment questions are already defined in a standard
 2057 form in the terms of reference and the assessment follows a standardised procedure.

2058 The evaluation of the biological relevance of an effect under consideration in the FEEDAP
 2059 Panel includes adverse (unwanted) effects and positive (wanted) effects on potentially
 2060 different species and has to address all aspects indicated above.

2061 The feed additive considered in this example consists of two essential oils derived from
 2062 steam distillation of *Thymus vulgaris* (thyme) and *Illicium verum* (star anise), quillaja
 2063 bark powder, crushed herbs and spices, and other feed materials (EFSA FEEDAP Panel,
 2064 2016). Thymol and *trans-anethole* are the major components of thyme oil and star anise
 2065 oil, respectively. Star anise oil may contain the alkenyl-benzene derivative estragole in
 2066 considerable concentrations.

2067 The additive is intended for use in chickens for fattening (the target species) at a dose of
 2068 150 mg/kg complete feed.

2069 Problem formulation at a glance is shown in the following Table.

Agents	Effects	Subjects	Conditions
Additive/Single active substances: <i>trans-anethole</i> 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-anethole</i>	Liver effects for <i>trans-anethole</i> in laboratory animals	Target species, Consumers	Dietary exposure via residues (data available from tolerance study, x10)
Single active substances: <i>trans-anethole</i> and thymol	Irritation, sensitisation skin	Users/workers	Exposure by inhalation, contact, systemic exposure
Single active substances: <i>trans-anethole</i> 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 Digestibility enhancer	Target species	Dietary exposure at the proposed use level

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 γ -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-sulfooxyestragole by SULT1A1 (Wiseman et al.,
2238 1985). Spontaneous abstraction of SO42- 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

2308 restricted to mice and occurs also in other species including birds and humans. The
2309 carcinogenicity of estragole in mice can therefore be taken as evidence for a possible
2310 carcinogenic effect of estragole in other species including humans.

2311 It was demonstrated that the percentage of 1-hydroxyestragole formed after application
2312 of estragole to mice and rats increases with the administered dose. At low
2313 concentrations, estragole is mainly metabolized via alternative pathways to non-
2314 genotoxic compounds (Anthony et al., 1987). Taken into consideration that the
2315 concentration of estragole residues in the tissue of chicken treated with the additive is
2316 very low, this fact increases the uncertainty that estragole residues represent a
2317 biologically relevant hazard for the consumer.

2318 Relevant adverse effects of trans-anethole and other non-genotoxic irritating compounds
2319 of the feed additive are restricted to the user/worker. These effects depend on the mode
2320 and level of exposure and thus to the safety precautions which are in place at working
2321 facilities.

2322 **Positive effect**

2323 The feed additive has the capacity to increase the performance of chicken for fattening,
2324 providing positive economic effect for the farmer. Any such effect exceeding the costs of
2325 the additive can be considered as relevant. However, the animal itself will not benefit
2326 from this positive effect.

2327 Considerable uncertainty exists about whether such effects might be attributed to certain
2328 compounds of the feed additive or they rather reflect an additive effect of the mixture.

2329 **References**

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2400 **Annex F – GMO**

2401 **Scientific Opinion on application EFSA-GMO-NL-2007-45 for the placing**
2402 **on the market of herbicide-tolerant, high-oleic acid, genetically modified**
2403 **soybean 305423 for food and feed uses, import and processing under**
2404 **Regulation (EC) No 1829/2003 from Pioneer**

2405 **EFSA Journal 2013; 11 (12): 3499**

2406 **Assessment strategy**

2407 The EFSA GMO Panel was requested to carry out a scientific assessment of soybean
2408 305423 (Unique Identifier DP-305423-1) for food and feed uses, import and processing
2409 in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003, i.e., to
2410 assess whether the import of Soybean 305423 and/or any of its derived products in the
2411 EU would result in additional safety concerns to animal and human health or to the
2412 environment with respect to conventional soybean.

2413 This application excludes cultivation in the European Union and the Environmental Risk
2414 Assessment is limited to the consequences of accidental spillage of imported soybeans
2415 and to the dissemination of faeces of animals feeding Soybean 305423.

2416 **Specification of the agent**

2417 1) The GM plant itself: Soybean 305423

2418 Soybean 305423 was transformed:

- 2419 • to express the *Glycine max-hra (gm-hra)* gene conferring tolerance to
2420 acetolactate synthase (ALS)-inhibiting herbicides;
- 2421 • to express a fragment of the endogenous *fad2-1* gene resulting, through RNA
2422 interference, in the silencing of the endogenous *fad2-1* gene, which leads to a
2423 decreased level of the omega-6 fatty acid desaturase and a high-oleic acid
2424 phenotype.

2426 Soybean 305423 can be present through a wide range of genetic backgrounds into which
2427 the event has been introduced through backcrosses from the original transformed line.

2428 2) Newly expressed protein: GM-HRA

2429 Two forms of this protein are considered:

- 2430 a. The GM-HRA protein expressed in soybean 305423
- 2431 b. The equivalent GM-HRA protein expressed in a recombinant microbial system (*E. coli*) to be used in toxicological studies.

2433 **Specification of the subjects**

- 2434 - Humans who are exposed to the agents through the consumption of soybean
2435 305423 oil or of food derived from soybean 305423;
- 2436 - Animal species that are exposed to the agents through the consumption of
2437 soybean 305423 derived feed.

2438 **Specification of the effects**

2439 For GMOs, the risk assessment framework refers to the identification and characterisation
2440 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.

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)

2506

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.
2550

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 glycinein**
 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-equivalence demonstrated or more likely than not). However, these differences were not considered biologically relevant for further safety assessment owing to their well-known 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 indication that the protein is allergenic (source, bioinformatics etc)**

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

2623 - *is it directly or indirectly linked to a(n) adverse/beneficial outcome? The potential effect 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 conventional comparators: NO EFFECT: no change in 2-D immunoblot patterns; no differences at ELISA analyses**

2629 - *Is the effect in itself adverse/positive: the potential effect could be negative; not applicable here*

2632 - *is it directly or indirectly linked to a(n) adverse/beneficial outcome? the potential effect 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 of the conventional counterpart and of non-GM reference varieties in its fatty acid profile, the newly expressed protein, the minerals zinc and calcium and the isoflavone glycinin. The variations in the fatty acid profile and the newly expressed protein are consistent with the objective of the modification as well as with the expression of the ALS enzyme of soybean 305423. A safety and nutritional assessment of the altered fatty acid profile and the newly expressed protein is provided in section 5 of the EFSA Scientific Opinion. For the remaining compounds, no further assessment was deemed necessary owing to their 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.

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

2695 done by measuring endpoints on subjects other than those used to test the agent. For
2696 example, in the GMO comparative analysis, commercial varieties are included in the field
2697 experiments, together with the GMO under assessment and its direct conventional
2698 counterpart. Statistical methods can be used to assess the observed differences against
2699 the natural variability observed among subjects not exposed to the agent. (Cf Statistical
2700 Guidance Document).

2701 Both difference and equivalence testing approaches are complementary: statistically
2702 significant differences may point at direct biological changes caused by the GM
2703 transformation, but that may not be relevant from the viewpoint of safety. On the other
2704 hand, equivalence assessments may identify differences that are potentially larger than
2705 normal natural variation. It should be pointed out that even if a difference is proven to
2706 fall within natural variation, it might still be relevant for further toxicological assessment
2707 if this change is observed consistently across subjects or if it may lead to
2708 indirect/carryover effects on the functioning of the biological system/ecosystem.

2709

2710 **References**

2711 EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2013.
2712 Scientific Opinion on application EFSA-GMO-NL-2007-45 for the placing on the
2713 market of herbicide-tolerant, high-oleic acid, genetically modified soybean
2714 305423 for food and feed uses, import and processing under Regulation (EC) No
2715 1829/2003 from Pioneer. EFSA Journal 2013;11(12):3499, 35 pp.
2716 doi:10.2903/j.efsa.2013.3499

DRAFT

2717 Annex G – NDA

2718 **Case study of biological relevance in the area of health claims:**
2719 **Application for substantiation of a health claim related to: Yestimun®**
2720 **and defence against pathogens in the upper respiratory tract (EFSA**
2721 **Journal 2013;11(4):3159)**

2722 Assessment strategy

2723 β -glucans are dietary fibres that have been shown to have immunomodulatory activity in
2724 animals and humans after oral administration. Common colds are caused by viruses,
2725 which are pathogens that are eliminated by the body's defence mechanisms. By virtue of
2726 the effect of β -glucans on the immune system, the fibres may support defence against
2727 pathogens in the upper respiratory tract.

2728 The food, which was the subject of the claim, that the NDA Panel was requested to
2729 evaluate, was Yestimun®, i.e. (1,3)-(1,6)- β -D-glucan produced from brewer's yeast cell
2730 wall (100 % *Saccharomyces cerevisiae*), and the claim was that this food would support
2731 the defence against pathogens in the upper respiratory tract.

2732 The NDA Panel needed to judge the claimed effect according a number of criteria:

- 2733 1. Specification of the agent: is the food sufficiently characterized to evaluate the
2734 claimed effect
- 2735 2. Specification of the subjects: Which is the target group for the claimed effect,
2736 and
- 2737 3. Identification: Is the claimed effect in itself a relevant health effect, i.e. is it
2738 biologically relevant, and
- 2739 4. Specification of the conditions: Is the information based on human intervention
2740 studies and other information. Are the studies, on which the applicant wants to
2741 base the claim of glucans supporting defence to respiratory infection, sufficiently
2742 powered and are measures performed according accepted standards and is
2743 statistical analysis done appropriately. As long as common cold could be
2744 attributed to infection with an infectious agent, such information would be useful.
2745 Information on other respiratory conditions that are not attributable to infection is
2746 not useful. Information on immune parameters, assessed in humans, animals, or
2747 in vitro systems, can only provide supportive or mechanistic information, but in
2748 the absence of a substantiated effect on defence do not provide any scientific
2749 evidence for the substantiation of the claim. Does a biological effect occur after
2750 ingestion of the glucan; reduction of an already evident infection is beyond the
2751 scope of health claims on food, such effects would be considered as therapeutic.

2752 Data Collection

2753 For the substantiation of the claim, three randomized controlled intervention studies were
2754 available. The primary endpoints were reduction in the number of common cold episodes
2755 per subject during the study periods, whereas secondary outcomes were severity of
2756 common cold episodes, duration of cold episode and the use of antibiotics and analgesics.
2757 The applicant provided information on the incidence of common cold.

2758 Data Evaluation

2759 The NDA Panel considered that the food was sufficiently characterized for the purpose of
2760 evaluating the claimed effect.

2761 In addition, the NDA Panel considers that supporting defence against pathogens in the
2762 upper respiratory tract, as measured by episodes of common cold, is in itself a beneficial
2763 physiological effect and therefore biologically relevant. In order to substantiate the claim,
2764 a statistically significant decrease in common cold episodes, if adequately proven with
2765 adequate confidence limits, would have been sufficient to substantiate the claim.

2766 For the substantiation of health claims, In the NDA Panel requires human data, notably
2767 intervention studies in humans. The target population is the general population and the
2768 subjects in the study, i.e. healthy individuals, represent the target population.

2769 The evidence provided did not establish the validity of questionnaires and criteria used to
2770 assess the incidence or the severity of common cold episodes. The power of the studies
2771 were likely adequate to observe effects on the primary endpoints. However, in one of the
2772 studies post-hoc analyses were performed based on episodes that occurred in the winter
2773 months (November to March, first half of the study) to avoid the potential error that
2774 might have arisen owing to possible misdiagnosis of allergic rhinitis as common cold
2775 infections during the summer months. Whereas this notion is understood, the post-hoc
2776 selection of the time windows for calculation of possible effects was not accepted as
2777 valid. In another study, statistical analysis did not account for the multi-centre design of
2778 the study.

2779 Overall conclusion

2780 In the judgement of the Panel, an effect on incidences of common cold, if appropriately
2781 shown to be statistically significant, would have been relevant for the purpose of
2782 substantiating the claim. However, even if statistically significant differences were
2783 reported, they were not judged relevant due to flaws in the statistical approach and the
2784 Panel came to the conclusion that a cause and effect relationship had not been shown.

2785 **Uncertainty**

2786 On the basis of the data presented, the NDA Panel concluded that a cause and effect
2787 relationship had not been established between the consumption of Yestimun® ((1,3)-
2788 (1,6)- β -D-glucans from brewer's yeast cell wall) and defence against pathogens in the
2789 upper respiratory tract. The opinion did not indicate that such a relation could not be
2790 there, but indicated that from the human studies provided conclusions could not be
2791 drawn.

2792 **References**

2793 EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2013.
2794 Scientific Opinion on the substantiation of a health claim related to Yestimun®
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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 D₂) and cholecalciferol (vitamin
2808 D₃), 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 D₂ and vitamin D₃ are present
2811 in foods and dietary supplements. Vitamin D₃ 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 D₃ 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**

2852 The next step was to assess the relationships between 25(OH)D and vitamin D intakes.
2853 In order to avoid confounding by endogenous synthesis, studies carried out during
2854 minimal sun exposure, lasting for at least 6 weeks, with oral exposure to vitamin D at
2855 least twice a week were considered. The articles which matched eligibility criteria were
2856 used to perform a quantitative analysis of the extracted data through a meta-analytic
2857 approach. Background intake was added to the supplemental vitamin D dose to generate
2858 total vitamin D estimates. When the habitual vitamin D intake was not reported,
2859 surrogates were imputed using appropriate age- and sex-specific mean vitamin D intake
2860 values (from food) from the national nutrition survey relevant to the country in which the
2861 study was performed.

2862 Two different models of the dose-response relationship between total vitamin D intake
2863 and plasma/serum 25(OH)D concentration were explored : a linear model and a non-
2864 linear model (i.e. with the natural logarithm transformation of the total intake). Finally,
2865 the Panel decided to retain the non-linear model to better describe the dose-response
2866 shape and to be able to include results from higher dose trials (*i.e* up to 50 µg/day).

2867 A number of factors potentially influencing the dose-response relationship were
2868 investigated, in order to select factors to be included in the final model to characterise
2869 the high heterogeneity of results across individual trials. After the inclusion of the final
2870 set of covariates, the adjusted R^2 (proportion of between-study variance explained) of
2871 the final model was 85 %, meaning that the fitted factors were able to characterise most
2872 of the across-trials variability in response. The models were used to predict the achieved
2873 mean serum 25(OH)D concentrations corresponding to total vitamin D intakes of 5, 10,
2874 15, 20, 50, 100 µg/day and to estimate the total vitamin D intakes that would achieve
2875 serum 25(OH)D concentrations of 50, 40, 30, 25 nmol/l.

2866 A number of sensitivity analyses were also carried out to evaluate whether the findings
2867 were robust to the assumptions made in the systematic review protocol and the
2868 analyses, in particular, on the background intake imputation process, on eligibility criteria
2869 (*e.g.* fortified food trials versus supplement trials); characteristics of participants (*e.g.*
2870 exclusion trials that did not explicitly exclude supplement users, persons with sun
2871 holidays, persons using sunbeds/artificial UV-B sources or going on sunny holidays).
2872 None of these sensitivity analyses raised serious concerns about the robustness of the
2873 overall analysis.

2874 The Panel considered that the results of this meta-regression analysis could be used to
2875 set DRVs for vitamin D.

2876 **Overall relevance taking into account the exposure**

2877 The Panel used information obtained from characterising the intake-status relationship
2878 for vitamin D to derive the vitamin D intake to achieve a target serum 25(OH)D
2879 concentration of 50 nmol/l. For the purpose of deriving AIs for vitamin D, the Panel
2880 decided to focus on the *adjusted* model obtained with data mostly on adults. The
2881 estimates from that model were derived based on all covariates.

2882 In the *adjusted model*, the total intake estimated to achieve a serum 25(OH)D
2883 concentration of 50 nmol/l, as identified by the lower limit of the 95% PI, is 16.1 µg/day.
2884 Equally, at a vitamin D intake of 15 µg/day, the predicted mean serum 25(OH)D
2885 concentration is 63 nmol/l (95 % CI: 58–69 nmol/l), with a predicted value at the lower
2886 limit of the 95% PI of 49 nmol/l.

2887 Predicted interval (PI) in the context of a meta-regression analysis illustrates the
2888 uncertainty about the true mean response predicted in a future study. Moreover, 95% PI
2889 constitutes an approximation of the interval that would include 95% of all individual
2890 responses from the populations included in previous and future studies, as it refers to the
2891 population of mean responses. The extent of this approximation could not be quantified.

2902 The Panel therefore set an AI for vitamin D for adults at 15 µg/day, considering that, at
2903 this intake, the majority of the adult population will achieve the target serum 25(OH)D
2904 concentration near or above 50 nmol/L. The Panel decided not to set specific AIs for
2905 'younger' or 'older' adults, because there was no evidence of a significant difference in
2906 absorption capacity between 'younger' and 'older' adults and the majority of the studies
2907 used to set the target value for 25(OH)D concentration were carried out in 'older adults'.

2908 The unadjusted model can be also taken into account as it encompasses the whole
2909 heterogeneity across trials. In the unadjusted model, considering a vitamin D intake of
2910 15 µg/day, the lower limit of the 95% PI is 34 nmol/L. This value is above the
2911 concentrations that have been observed in relation to overt adverse health outcomes
2912 (osteomalacia, calcium absorption). Considering a vitamin D intake of 15 µg/day, the
2913 upper limit of the 95% PI is 91 nmol/L in the unadjusted model (and 78 nmol/L in the
2914 adjusted model). These values are in the physiological range.

2915 **References**

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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/EC² 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.

² <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

3004 that have presence of this nematode but can import under certain restrictions) are
3005 considered, and those within Europe. Furthermore, information is needed on the
3006 prevalence of the nematode in the trade flows. Furthermore, information is needed on
3007 the host plants of the nematode, and the impact caused on those hosts. In addition
3008 information is needed on the conditions for establishment. The panel focused the
3009 assessment on seed potato and flower bulbs, which constitute the most important plant
3010 product that can serve as a vector. Furthermore, ware potatoes and many flower bulb
3011 species are hosts and can suffer damage.

3012 **Data evaluation**

3013 Adequate data are available on the international trade in seed potatoes. However, no
3014 adequate data are available on the trade in flower bulbs because the data in Eurostat are
3015 not recorded at a sufficient level of resolution between species. Therefore, trade in host
3016 species and non-host species cannot be well distinguished. Data on production areas of
3017 seed potatoes across the EU are good. Data on production areas of flower bulbs are
3018 adequate.

3019 Information on the prevalence of the nematode in the EU and in third countries is
3020 extremely sparse. Only vague descriptions are available like "present in all parts of the
3021 area where host crops are grown", "present, restricted distribution", "present, few
3022 occurrences", "present", and "absent". These terms were interpreted by the panel in
3023 terms of proportion of production fields infested with the nematode and proportion of
3024 planting material harvested from infested fields that carry the nematode. This
3025 interpretation is a reason for large uncertainties in the estimates of the flow of infested
3026 planting material. Furthermore, the panel made assessments of survival of the nematode
3027 in trade flows, and of the efficacy of import and export inspection and of certification
3028 schemes to reduce or limit the levels of infestation of plant product with the nematode.
3029 Due to lack of pertinent data, these assessments were also quite uncertain.

3030 **Relevance of the agent**

3031 The potato rot nematode *Ditylenchus destructor* causes rots in root crops and bulbous
3032 crops. The species is well characterized and the potential damaging effects are also well
3033 characterized. If uncontrolled, this nematode can multiply, spread and cause substantial
3034 damage. However, with current phytosanitary measures for containing the spread with
3035 plant products, and efficient weed control in crops, reducing the number of potential
3036 hosts for the nematode, the impact of this nematode under current conditions is minor.

3037 Nematodes can live in tubers, bulbs and rhizomes, and they can be spread with trade in
3038 such products over practically unlimited distance. Infested planting material is the main
3039 pathway for spread. Autonomous spread by nematodes is not practically relevant. Spread
3040 by farm machinery is possible but is still a short distance spread (mostly within field or
3041 farm). There is no known minimum number of nematodes that is required to cause
3042 establishment or infestation of a plant.

3043 **Relevance of the subject**

3044 The panel made the assessment focusing on seed potatoes and tulip bulbs. Seed
3045 potatoes are the most important carrier for the nematode. Flower bulbs are also
3046 potentially important, and the trade in the host species tulip is the largest amongst
3047 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. In stead 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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3090 *Ditylenchus destructor* for the EU territory. To go for adoption in September 2016.

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3092 Annex I – PPR**3093 PESTICIDES HAVING EFFECTS ON THE THYROID HORMONE SYSTEM**

3094 In the MRL regulation 396/2005 it was laid down that account should be taken of "the
3095 possible presence of pesticide residues arising from sources other than current plant
3096 protection uses of active substances, and their known cumulative and synergistic effects,
3097 when the methods to assess such effects are available".

3098 Then in the later pesticide regulation 1107/2009, the precautionary principle applies and
3099 therefore before placing active substances in plant protection products on the market it
3100 should be demonstrated that they do not have any harmful effect on humans.

3101 However, not only the single active substance should not have harmful effects but should
3102 also take account of effects from mixtures of pesticides. Thus, the regulation reads "shall
3103 have no immediate or delayed harmful effect on human health, including that of
3104 vulnerable groups, or animal health, directly or through drinking water (taking into
3105 account substances resulting from water treatment), food, feed or air, or consequences
3106 in the workplace or through other indirect effects, taking into account known cumulative
3107 and synergistic effects..."

3108 This would be applicable not only for dietary risk assessment but also non-dietary
3109 (operators, workers, bystanders and residents).

3110 EFSA was in accordance with the regulation commissioned to develop the methodology
3111 for carrying out cumulative risk assessment in regard to MRL-setting and launched this
3112 work in 2006 with a scientific colloquium followed by subsequent opinions, amongst the
3113 "Scientific opinion on the identification of pesticides to be included in cumulative
3114 assessment groups on the basis of their toxicological profile". In the opinion a
3115 methodology for grouping was developed and two cases where elaborated on; Pesticides
3116 having effects on the nervous system and pesticides having effects on the thyroid
3117 system. A total of nearly 300 pesticide dossiers were evaluated for these two cases.

3118 Assessment strategy

3119 In this case, two problem formulations are being answered on the basis of the same
3120 data-sets.

- 3121 1. For the single pesticidal active substance assessment, establish no observed
3122 adverse effect levels (NOAELs) in case of effects on the thyroid system.
- 3123 2. Identification of pesticides to be included in cumulative assessment groups
3124 (CAG's) on the basis of their toxicological profile (hazard assessment) – in this
3125 case effects on the thyroid system – and establishing NO(A)EL's in this context.

3127 The establishment of the critical effect, the NOAEL for this effect and deriving reference
3128 doses (AOEL, ADI, ARfD) is to protect the human population exposed to the pesticide
3129 when applied and to the protect the population being exposed via all routes of exposure.

3130 The grouping of the pesticides into CAG's was developed to support the regulatory MRL-
3131 setting and as such the target population is the European Consumer.

3132

Agents	Effects	Subjects	Conditions
Single active substance		The human population	Dietary and non-dietary exposure. Establishment of NOAEL in subchronic – chronic exposure.
Cumulative Assessment Groups of active substances (CAGs)	Effects on the thyroid system	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

3158 considered that the statistical significance in the dog studies might not be reliable due to
 3159 the high inter-individual variability.
 3160

3161 Relevance of the effects

3162 For detailed description of the thyroid system, see Miller (2009) and the following figure
 3163 showing where chemicals might perturbate the thyroid system.

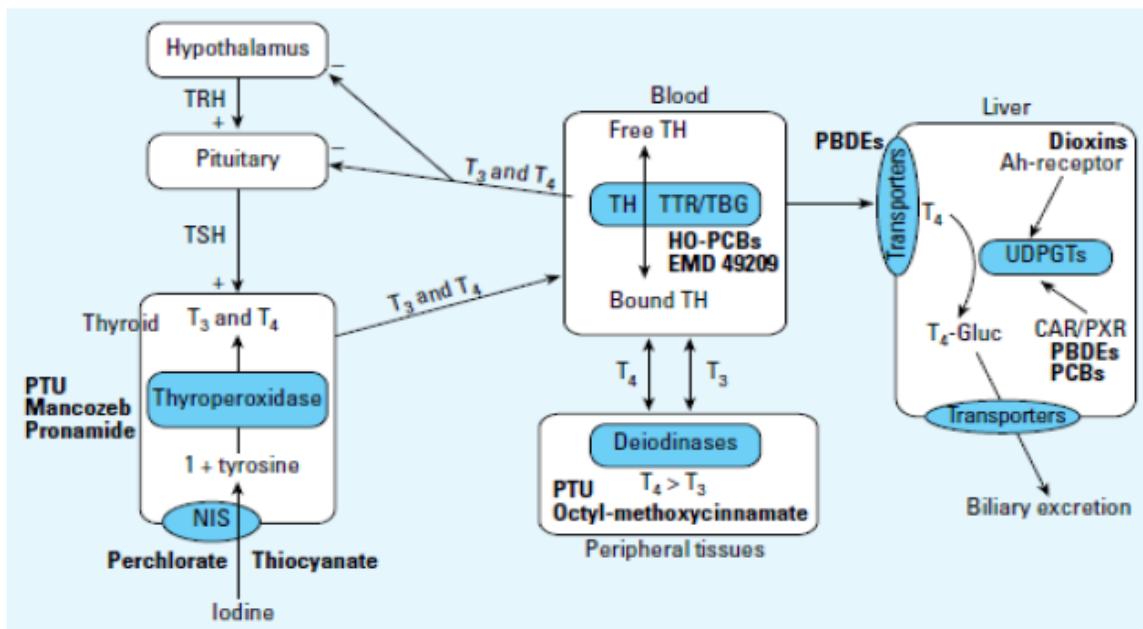


Figure 1: Thyroid hormone system with potential targets for disruption by xenobiotics (blue). NIS, sodium/iodide symporter; TBG, thyroid hormone-binding globulin; TH, thyroid hormone (T_3/T_4); T_3 , triiodothyronine; T_4 , thyroxine; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; TTR, transthyretin; UDPGTs, UDP-glucuronosyl transferases (Miller et al., 2009).

3164
 3165 When declines in circulating and tissue hormone levels occur, feed-back mechanisms of
 3166 the hypothalamic-pituitary-thyroid axis would result in increased secretion of TSH
 3167 (Thyroid Stimulating Hormone) and subsequently the follicular cells would increase the
 3168 secretion of T_3/T_4 and thus levels of bio-available T_3/T_4 would be re-adjusted.

3169 Relevance of thyroid system disruption to humans

3170 Alterations in circulating bioavailable thyroid hormone levels may have serious impact on
 3171 other organs or organ systems besides the thyroid itself also in humans, particularly if
 3172 perturbations occur during critical windows of development" (EFSA 2013).

3173 And further the PPR panel noted that "Any degree of thyroid disruption that lowers TH
 3174 levels on a population basis should be considered a biomarker of increased risk of
 3175 adverse outcomes, which may have important societal outcomes" (Miller et al., 2009)
 3176 (EFSA 2013)". So as such adverse effects/effects on the thyroid system are considered
 3177 relevant for the human population.

3178

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.

3246 References:

3247 EFSA Journal, 2013; 11(7): 3293. Scientific opinion on the identification of pesticides to
3248 be included in cumulative assessment groups on the basis of their toxicological profile.

3249 Miller MD, et al. Thyroid-disrupting chemicals: interpreting upstream biomarkers of
3250 adverse outcomes. EHP 117; 1033-1041.

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3261 **Annex J – Chemical Risk Assessment**

3262 **Human health risk assessment of Cadmium in food:**

3263 **Scientific Opinion of the Panel on Contaminants in the Food Chain; The**
 3264 **EFSA Journal (2009) 980, 1-139.**

3265 **Assessment strategy**

3266 Cadmium (Cd) is a heavy metal found as an environmental contaminant, both through
 3267 natural occurrence and from industrial and agricultural sources. Foodstuffs are the main
 3268 source of cadmium exposure for the non-smoking general population. Cadmium
 3269 absorption after dietary exposure in humans is relatively low (3–5 %) but cadmium is
 3270 efficiently retained in the kidney and liver in the human body, with a very long biological
 3271 half-life ranging from 10 to 30 years.

3272 For the purpose of the guidance and although the scientific assessment of cadmium had
 3273 a broader content, the example below focuses only on one effect.

3274 The kidney is the critical target organ for dietary exposure to cadmium and renal damage
 3275 is characterised by cadmium accumulation in convoluted proximal tubules, thereby
 3276 causing cell dysfunction and damage. The earliest signs of tubular toxicity are
 3277 respectively decreased tubular reabsorption (increased excretion) of low molecular
 3278 weight proteins (LMWP) and increased excretion of markers of cell shedding.

3279 Problem: Characterise critical effect for the purpose of deriving a Health based guidance
 3280 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)

3281

3282 **Data collection/ Data evaluation for each dataset**

3283 The availability of quantitative human data for both toxicokinetics (TK) and
 3284 toxicodynamics (TD) provides relevant data for hazard identification and characterisation
 3285 without the need to use animal data.

3286 **Data Evaluation (Biological Relevance)**

3287 Cadmium is bio-accumulating due to very slow renal excretion (TK), leading to excretion
 3288 of biomarkers of kidney damage (TD).

3289 **Biological Relevance of biomarker of proximal tubular dysfunction**

3290 **Is the effect in itself a(n) adverse/positive effect?**

3291 The CONTAM Panel based its assessment on the use of the low molecular weight protein
 3292 (LMWP) beta-2-microglobuline (B2M) in urine as biomarker of Cd-induced tubular
 3293 toxicity. Increased excretion of B2M is not per se associated with any objective symptom
 3294 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 ^{a)} 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

^{a)} 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 g 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.

3333 To account for inter-individual variations in cadmium concentration within groups, not
3334 explicitly accounted for in the BMD modelling (i.e. when calculating the lower one-sided
3335 95 %-confidence bound for an extra risk of 5 % of producing a specified change in the
3336 urinary level of the B2M, denoted BMDL5), the CONTAM Panel modified the BMDL5 value
3337 using a chemical specific adjustment factor (CSAF) for cadmium based on the estimated
3338 variance of within group cadmium concentration. After adjustment, the CONTAM Panel
3339 identified a critical cadmium concentration of 1 µg cadmium/g creatinine in urine as a
3340 modified reference point (RP) on which to base a health based guidance value (HBGV) of
3341 cadmium dietary intake.

3342 **Converting the RP to an intake value and derive a HBGV for Cd**

3343 Subsequently, a one-compartment population toxicokinetic (TK) model was fitted to 680
3344 paired data of cadmium intake and urinary cadmium concentrations from the Swedish
3345 Mammography Cohort study (Amzal et al., 2009). This TK model showed that a dietary
3346 intake of no greater than about 2.5 µg/kg b.w. cadmium per week would prevent 95 %
3347 of the Caucasian population from being above the modified RP of 1 µg cadmium/g
3348 creatinine in urine after 50 years of exposure (EFSA, 2009a). In order to remain below
3349 this modified RP it was calculated that the average daily dietary cadmium intake should
3350 not exceed 0.36 µg/kg b.w., and this daily intake was used to derive the TWI of 2.5
3351 µg/kg b.w.

3352 **References**

3353 Scientific Opinion of the Panel on Contaminants in the Food Chain on a request
3354 from the European Commission on cadmium in food. The EFSA Journal (2009)
3355 980, 1–139.

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3356 Annex K – Environmental Risk Assessment

3357 In environmental risk assessment the protection goal is normally based on protecting
3358 populations. In some cases, it is also based on individuals, for instance for all
3359 vertebrates. Sometimes the protection goal is a function, for instance nitrification, and
3360 sometimes it includes even behaviour e.g. for bees and vertebrates.

3361 For example, in the Avian Reproductive Test (OECD 206) the following endpoints must be
3362 assessed:

- 3363 • Frequency, duration and description of signs of toxicity, along with severity,
3364 numbers affected and any remissions
- 3365 • **Food consumption** and **body weight** for adults and juveniles
- 3366 • Details of gross pathological examinations
- 3367 • Results of residue analysis (if performed)
- 3368 • **Egg production** – number of eggs laid per hen (10 weeks)
- 3369 • Percentage of **cracked eggs**
- 3370 • **Viability** (per cent viable embryos of eggs set)
- 3371 • **Hatchability** (per cent hatching of eggs set)
- 3372 • **Percentage of hatchlings** that survive to 14 days
- 3373 • Number of **14 day old survivors** per hen
- 3374 • **Eggshell thickness** (mm)

3375 The test should be carried out with a **minimum** of three dietary concentrations of the test
3376 substance. The concentrations to be used should be based upon the results of a dietary
3377 LC50 test (OECD 205). The highest concentration should approximately be one half of
3378 the LC10. Lower concentrations should be geometrically spaced at fractions of the
3379 highest dose (e.g. 1/6 and 1/36 of the highest dose).

3380 As a consequence of this design the power for each endpoint is different. For some
3381 endpoints the power will be very weak and some others strong and they will vary
3382 between compounds and over time.

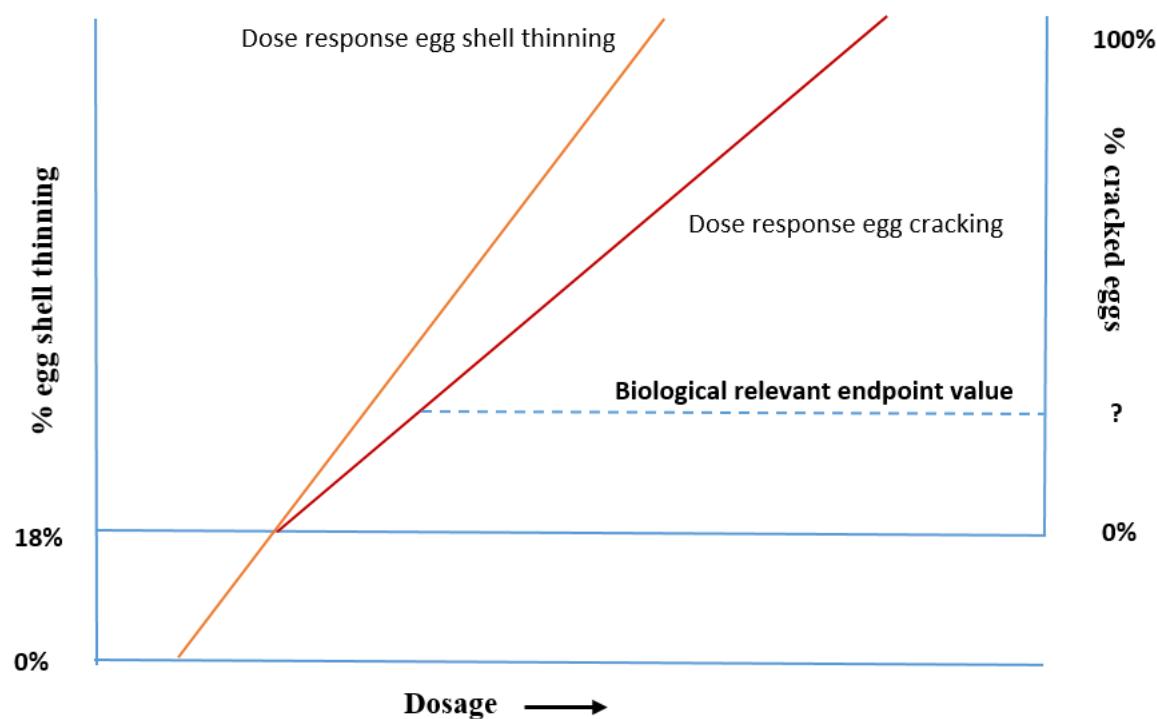
3383 All endpoints are in principle assumed to be relevant when populations are the protection
3384 goal. The hazard assessment of the bold printed endpoints above is based on a NOEC,
3385 i.e. the highest tested concentration in which the values for the observed effect are not
3386 significant different from the control. Of all other observations the risk assessor has to
3387 consider whether these effects could influence the survival of the population.

3388 Although an endpoint is statistically significantly different from the control it may not be
3389 biologically relevant. In order to determine the biological relevance of an effect it should
3390 be considered whether the effect could lead to a functional deficit later on in the study,
3391 e.g. if a reduction in the weight of pups at birth leads to a decrease in level of survival. If
3392 not, then the effect may not be biologically relevant, however if there is a carry-over of
3393 effects into the number of survivors, it can be considered biologically relevant.

3394 **Example involving egg shell thickness and cracked eggs**

3395 As stated above not all outcomes of the test are biological relevant, for instance if the
3396 LOEC (lowest observed effect concentrations) for egg shell thinning is 3% than it is
3397 generally believed that the NOEC does not have a biological relevance. It is believed that
3398 the biological relevant percentage of egg shell thinning starts with 18% (Blus 2003, EFSA

3399 2009). The BMD dose equivalent to 18% effect can be calculated with an appropriate
 3400 method. This BMD can be considered as the "NOEC" for cracked eggs (see figure below).



3401

3402 **Figure 5: Relation between egg shell thickness (orange line) and cracked eggs (red line). The dashed line is the line for**
 3403 **effecting the reproduction of a bird species (e.g. when is the number of cracked eggs too much for maintaining a stable**
 3404 **population).**

3405 In many cases it will be difficult to point out what the biological relevant threshold of an
 3406 endpoint will be. A tool that can be used is to run legislative acceptable models and to
 3407 assess at which percentage a population will not be able to recover any more or when a
 3408 population suffers to an unacceptable degree and to include in this assessment the
 3409 uncertainty around the outcome.

3410 A "NOEC" for cracked eggs is in view of a precautionary principle a good starting point for
 3411 the risk assessment but it is probably not the value at which a population will start to
 3412 show signs of decreased ability to survive.

3413 Another approach is to include recovery in the risk assessment, which is for instance an
 3414 option in aquatic risk assessment (not fish or amphibians) and terrestrial risk assessment
 3415 for invertebrates. In the aquatic ecosystem a compound can be allowed on the market
 3416 when recovery is seen within a period of 8 weeks but only when all important organism
 3417 groups are included in the mesocosm experiment (see opinion on recovery, EFSA 2013
 3418 and 2016).

3419 **References**

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3425 organisms in edge-of-field surface waters. EFSA Journal 2013;11(7):3290, 268 pp.
3426 doi:10.2903/j.efsa.2013.3290.

3427 EFSA 2016. Scientific opinion on recovery in environmental risk assessments at EFSA.
3428 EFSA Journal 2016; 14(2):4313. 85 pp. doi:10.2903/j.efsa.2016.4313.

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3452 **Glossary [and/or] abbreviations (To be completed in the final**
3453 **document)**

3454 Toxicokinetics (TK): Describes the processes leading to the internal concentrations of a
3455 chemical or its metabolites(s) through knowledge of absorption (A), distribution (D),
3456 metabolism (M) and excretion E (ADME).

3457 Toxicodynamics (TD): Describes the processes that lead to the toxic effects of a chemical
3458 or its metabolites(s) once it has reached the organ(s) or tissue(s).

3459 Reliability: Refers to the degree to which an assessment tool produces stable and
3460 consistent results. (<https://www.uni.edu/chfasoa/reliabilityandvalidity.htm>).

3461 Protection goals: Natural resources (e.g. arthropod natural enemies, bees) or
3462 natural resource services (e.g. regulation of arthropod pest populations,
3463 pollination) that are to be protected as set out by EU.

3464 Confounding (Taken from Wikipedia, <https://en.wikipedia.org/wiki/Confounding>)

3465 In statistics, a confounding variable (also confounding factor, a confound, a lurking
3466 variable or a confounder) is an extraneous variable in a statistical model that correlates
3467 (directly or inversely) with both the dependent variable and the independent variable, in
3468 a way that "explains away" some or all of the correlation between these two variables.

3469 Bradford Hill criteria (Taken from Wikipedia,
3470 https://en.wikipedia.org/wiki/Bradford_Hill_criteria)

3471 (Hill, Austin Bradford. 1965. The Environment and Disease: Association or
3472 Causation? Proceedings of the Royal Society of Medicine. 58; 5: 295–300.)

3473 The Bradford Hill criteria, otherwise known as Hill's criteria for causation, are a group of
3474 guidelines that can be useful for providing evidence of a causal relationship between a
3475 putative cause and an effect, established by the English epidemiologist Sir Austin
3476 Bradford Hill (1897–1991) in 1965.

3477 The list of the criteria is as follows:

3478 1. Strength (effect size): A small association does not mean that there is not a causal
3479 effect, though the larger the association, the more likely that it is causal.[1]

3480 2. Consistency (reproducibility): Consistent findings observed by different persons in
3481 different places with different samples strengthens the likelihood of an effect.[1]

3482 3. Specificity: Causation is likely if there is a very specific population at a specific site and
3483 disease with no other likely explanation. The more specific an association between a
3484 factor and an effect is, the bigger the probability of a causal relationship.[1]

3485 4. Temporality: The effect has to occur after the cause (and if there is an expected delay
3486 between the cause and expected effect, then the effect must occur after that delay).[1]

3487 5. Biological gradient: Greater exposure should generally lead to greater incidence of the
3488 effect. However, in some cases, the mere presence of the factor can trigger the effect. In
3489 other cases, an inverse proportion is observed: greater exposure leads to lower
3490 incidence.[1]

3491 6. Plausibility: A plausible mechanism between cause and effect is helpful (but Hill noted
3492 that knowledge of the mechanism is limited by current knowledge).[1]

3493 7. Coherence: Coherence between epidemiological and laboratory findings increases the
3494 likelihood of an effect. However, Hill noted that "... lack of such [laboratory] evidence
3495 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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