TOX/2021/04

COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Second draft statement on the potential risks of combined exposure to mycotoxins

Background

1. The potential risks from combined exposure to mycotoxins was identified as a topic that the Committee on Toxicity in Food, Consumer Products and the Environment (COT) should consider during horizon scanning.

2. A preliminary scoping paper regarding the potential risks from combined dietary exposure to mycotoxins (TOX/2020/34)[1] was reviewed by the COT in July 2020. Since then, the topic and additional information has been discussed several times by the COT with the final substantive discussion in October 2020.

3. The draft statement as presented in Annex A brings together the conclusions from these discussions and lists the research recommended by the COT.

Questions on which the views of the Committee are sought

4. Members are invited to consider the following questions:

   i). Are Members happy with the changes made?

   ii). Are there any other aspects that have been addressed during the COT review of combined exposure to mycotoxins that are not covered in the second draft statement which should be included?

   iii). Do the conclusions accurately represent the views of the COT?

   iv). Does the Committee have any other comments?

Secretariat
February 2021

[1] TOX/2020/34 is available on the COT website.

COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Second draft statement on the potential risks of combined exposure to mycotoxins

Background

1. The COT horizon scanning identified consideration of potential risks from combined exposure to mycotoxins as a priority.

2. Climate change could have a significant impact on the life cycle stages[2] and rates of the development of toxicogenic fungi which has the ability to modify host-resistance and host-pathogen interactions. Changes in the climate are expected to affect levels of rainfall, humidity, temperature *etc.* which in turn, will influence the conditions for mycotoxin production that varies for each individual pathogen species and/or strain.

3. Furthermore, advances in analytical techniques have allowed the simultaneous detection and quantification of multiple mycotoxins in both food and feed commodities (Krska *et al*., 2007; De Santis *et al*., 2017; Flores-Flores & González-Peñas, 2017; Bessaire *et al*., 2019; Singh & Mehta, 2020; Agriopoulou *et al*., 2020).

4. In light of this, new combinations of factors (mycotoxins/host plants and geographical location) will need to be considered when assessing the potential risks of combined exposure to mycotoxins.

Introduction

5. Mycotoxins are toxic secondary metabolites produced by fungi and can cause adverse health effects in both humans and animals. Cereals are often the crops most severely affected; however, some nuts, fruits and spices can

[2] There are four main stages for the fungi life cycle; hyphal growth, spore formation, spore dispersal and spore germination.

also be affected. Mycotoxins are stable low-molecular weight chemicals and not often affected by food processing.

6. The mycotoxins of greatest concern to human health and livestock are produced by several genera of filamentous fungi, namely *Aspergillus*, *Fusarium* and *Penicillium*, which produce aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT), fumonisins (FBs), zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), citrinin (CIT), T-2 and HT-2 toxins (WHO, 2018).

7. Exposure to dietary mycotoxins can lead to various adverse effects in humans, which include carcinogenic, teratogenic, hepatotoxic, nephrotoxic, and cytotoxic, immunological and haematological effects.

8. In addition to primary mycotoxins, modified mycotoxins can also be produced by fungi or generated as part of the defence mechanism of the infected plant. They are described as metabolites and normally remain undetected during the testing for parent mycotoxins (*e.g.* deoxynivalenol-3-glucoside when testing for DON). It has been reported that some modified mycotoxins can be converted into the parent mycotoxin during digestion in humans and animals, and thus have the potential to result in higher exposure to the parent mycotoxin and consequently increase the likelihood of adverse health effects. Although, the toxicological data for this is scarce (Freire & Sant'Ana, 2018).

Prevalence and co-occurrence

9. DON, FBs, and ZEN are the most prevalent mycotoxins in the world, with a prevalence of 66%, 56%, and 53%, respectively in cereals and cereal based products (Smith, 2016).

10. The production of mycotoxins can occur pre-, during or post-harvest. Several factors can influence the production of mycotoxins pre-harvest, such as the sowing time, plant density, soil conditions, irrigation, presence of weeds and pests. During harvest, the influencing factors include drying, cleaning and sorting of the crops, whereas the post-harvest factors are mainly associated with storage and processing. Mycotoxin colonisation also depends on the temperature, relative humidity, rainfall and water activity[3] at each of these stages. The presence of the fungi spores in crops does not always result in the production of mycotoxins, since optimal growth conditions are required for biosynthesis (Battilani *et al*., 2020).

11. The natural co-occurrence of mycotoxins in food and feed is quite common and occurs for three main reasons; (i) some fungi can produce more than one mycotoxin (particularly *Fusarium* spp.), (ii) food commodities can be contaminated by several fungi and (iii) animal and human diets usually consist of multiple commodities.

[3] Water activity is a measure of the availability in a substrate of water for microbial growth.

12. At present, there is limited availability on data with regards to co-occurrence of mycotoxins in food commodities especially in Europe. In contrast, worldwide surveys for mycotoxins in feed are more common of which, the BIOMIN Mycotoxin Survey[4] is one of the most comprehensive.

13. Updated results for January – September 2020 on the occurrence of mycotoxins in ~15,500 finished feed and raw commodity samples from 74 countries based on ~68,700 analyses were published in November 2020. It was observed that mycotoxins are rarely found alone; multiple mycotoxins co-contaminated feed materials (68% of all samples analysed for at least two mycotoxins). Results specific for Northern Europe shows high contamination of DON at an average of 485 ppb in straw (BIOMIN, 2020a). *Table 1* provides a mycotoxin prevalence breakdown for Central, Eastern, Northern and Southern Europe.

Table 1 - the BIOMIN Mycotoxin Survey Central, Eastern, Northern and Southern Europe results on prevalence of mycotoxins in animal feed (%) for January to September 2020 (reproduced from BIOMIN, 2020b).

Mycotoxin	**AF**	**ZEN**	**DON**	**T-2**	**FBs**	**OTA**
Central Europe	8	47	64	27	48	10
Eastern Europe	4	41	37	43	32	31
Northern Europe	1	31	58	25	24	7
Southern Europe	12	46	58	13	90	14

Abbreviations: AF = Aflatoxins; ZEN = Zearalenone; DON = Deoxynivalenol; T-2 = T-2 toxin; FBs = Fumonisins; OTA = Ochratoxin-A.

14. Some publications note that there is still limited knowledge on the presence and co-occurrence of multiple mycotoxins, both for primary mycotoxins and their modified forms, in food and feed (Palumbo *et al*., 2020), and that it is difficult to infer trends or recent developments regarding mycotoxin contamination in European feed from available data due to the influence of the respective cropping season's climate on the contamination levels which caused high year to year variation of results, as well as the differences in the applied analytical methods used to detect the contamination (Streit *et al*., 2012).

[4] The BIOMIN Mycotoxin Survey constitutes the longest running and most comprehensive survey of its kind. The survey results provide insights on the incidence of the six major mycotoxins (AFs, ZEN, DON, FBs, T-2 and OTA) in the agricultural commodities used for livestock feed in order to identify the potential risk posed to livestock animal production. Further information available on the BIOMIN website.

Methods for sampling and measuring mixtures of mycotoxins in food matrices

15. The main analytical methods to measure mycotoxins are enzyme-linked immunosorbent assay (ELISA), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). Currently, LC based methods are the most frequently used (Serrano *et al*., 2012; Malachová *et al*., 2018), with several mass spectrometric detectors such as single-quadrupole mass spectrometer, time-of-flight, triple-quadrupole, ion trap and orbital ion trap mass analysers, as well as hybrid systems that combine two types of analysers (*e.g.* fluorescence detector with LC systems).

16. An important and critical step is sample preparation and clean-up with techniques including solid phase extraction, matrix solid-phase dispersion, liquid–liquid and solid–liquid partitioning, accelerated solvent extraction, multifunctional columns and immunoaffinity columns (Serrano et al., 2012; Agriopoulou *et al*., 2020).

17. The main analytical method used for detecting and measuring co-occurrence of very low concentrations of mycotoxins is liquid chromatography tandem mass spectrometry (LC–MS/MS) (Serrano *et al*., 2012; Gambacorta *et al*., 2018; Malachová *et al*., 2018; Shi *et al*., 2019; Battliani *et al*., 2020; Palumbo *et al*., 2020); however, multi-mycotoxins analyses are not widely performed due to their associated high cost.

Current state of authoritative assessment and research

18. The COT have reviewed the European Food Safety Authority (EFSA) external report on mycotoxin mixtures in food and feed (Battiliani *et al*., 2020), several opinions by authoritative groups on some mycotoxin combinations, the work produced by the Mycotoxin mixtures (MYCOMIX) project led by Paula Alvito and her colleagues, as well as being aware of the Mycotoxin and Toxigenic Moulds (MYTOX) research group co-ordinated by Sarah De Saeger; as presented in TOX/2020/34[5]. Brief summaries are provided below.

European Food Safety Authority (EFSA) external scientific report on mycotoxin mixtures

19. In this EFSA report, titled "*Mycotoxin Mixtures in Food and Feed: Holistic, Innovative, Flexible Risk Assessment Modelling Approach*" (MYCHIF); Battilani *et al*., (2020) performed an extensive literature review out across four topics relating to the investigation of mycotoxin mixtures present in food and feed. These topics were:

[5] TOX/2020/34 available on the COT website.

i). Ecology and interaction with host plants of mycotoxin producing fungi, mycotoxin production, recent developments in mitigation actions of mycotoxins in crop chains;
ii). Analytical methods for primary, modified and co-occurring mycotoxins;
iii). Toxicity, toxicokinetics (TK), toxicodynamics (TD) and biomarkers relevant to humans and animals and;
iv). Modelling approaches, and key reference values for exposure, hazard and risk modelling.

20. The data collected from these were then stored in the MYCHIF platform hosted by EFSA. The main objective of which was to develop an integrated method supported by modelling, for the risk assessment of mycotoxin mixtures in food and feed. Each topic will be summarised in the following paragraphs.

21. It was observed that *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp., were the most relevant mycotoxins worldwide, but this can also be extended to *Alternaria* spp. and *Clavicleps* spp. to a minor extent. The production of mycotoxigenic fungi are not commonly host specific, since their occurrence is mainly associated with a specific crop depending on its region of growth and meteorological conditions. A fungus can produce different types of mycotoxins (e.g. sterigmatocystin which is a precursor for AFs), as such, contamination of food and feed stuffs can occur concurrently. Modified mycotoxins may also co-occur with primary varieties as a result of fungi-host plant interaction or during processing.

22. The MYCHIF report deemed that temperature, relative humidity, rainfall, are the most important ecological factors that influence fungal colonisation of substrates. Additionally, each species would have its own ecological needs and requirements. In general, mycotoxins are stable compounds and can accumulate over time (both during crop growth and post-harvest). Therefore, mitigation of contamination requires both good practices at all production stages.

23. In terms of the methodologies used for mycotoxin analysis, these are split into two categories. Firstly, screening tests provide qualitative or semi-quantitative results. They are generally based on antibody recognition and these methods are often relatively straightforward to carry out. The other category is confirmatory analysis which provides confirmation of fungal species identity and quantitative results. The most widely used quantification method is High Performance Liquid Chromatography (HPLC). LC-MS is also used to identify and quantify mycotoxins. A number of high- or ultra-chromatography coupled to mass spectrometry systems have the ability to measure both regulated mycotoxins and other lesser tested for mycotoxins with analytical standards available (*e.g*. CIT, sterigmatocystin *etc.,*) together in different feed food commodities; however, there are some limitations in these systems including: cost, sensitivity to include lower limits of quantification and

detection of *in vivo* metabolites, and a harmonised fit for purpose methodology characterised by the ability to measure multiple mycotoxins.

24. Toxicity, TK and TD parameters for humans and animals were collected from the literature to build three databases in the MYCHIF platform. These are: *in vitro* toxicokinetic data, *in vivo* toxicokinetic data, and *in vivo* toxicity data. A limited number of articles on mixtures were observed in comparison to those only exploring effects of single compounds. The information available only covers a limited number of combinations of mycotoxins. TK data were mainly reported in pigs and chickens, and rats. TD modelling of mycotoxin mixtures could not be performed using the currently available data.

25. The mycotoxin dose, exposure pathway, interspecies and intraspecies differences were identified to be the most important parameters that may influence toxicokinetic parameters.

26. As part of the MYCHIF project, it was highlighted that testing of all mycotoxin mixture combinations was not feasible, and as such, focus should be given on the prioritisation of mycotoxin mixtures, the creation of harmonised methods for generating *in vitro* and if required additional *in vivo* toxicokinetic data, and utilising predictive kinetic modelling (that includes uncertainty, and inter- and intraspecies variability analysis).

27. For the exposure assessment, human biomonitoring data was collected from the literature; 66/176 articles that focused on biomarker studies of multi-mycotoxins were selected for further analysis by Battilani *et al*., (2020). A multi-biomarker study was defined whereby both the parent and one or more metabolite were measured. Regarding biomonitoring in humans, AFs is the most widely studied mycotoxin followed by OTA, DON, FBs, ZEN and other emerging mycotoxins such as alternaria (ALT), tenuazonic acid, fusarenon-X (Fus-X,) neosolaniol, CIT, NIV, T-2, 4,15-diacetoxyscirpenol (4,15-DAS), and enniatins (ENNs) in a very few studies. The most common sample matrix was urine, followed by serum, plasma, blood, breast milk, colostrum and amniotic fluid.

28. The simultaneous determination of more than one mycotoxin in human biological fluids presents as a new challenge in mycotoxin biomonitoring. There are several constraints; in an analytical context, there is a lack of method standardisation and the unavailability of commercial reference standards (especially glucuronides). Their use in exposure assessments may be premature and cannot be fully exploited since there is a lack of: knowledge of effects of different combinations on the bioavailability of each individual component, the excretion rate, and a consensus of a validated biomarker to be used in context to a multi-mycotoxin analysis.

29. To paraphrase, there is still a lack of harmonisation in the experimental settings (*e.g.* for the use and validation of analytical methods) and design of biomonitoring studies (*e.g.* the selection of a candidate biomarker), in the data collection and in the definition of performance of fit for purpose analytical

methods. These highlighted issues meant it was not possible for Battilani *et al*., (2020) to exploit the biomonitoring dataset for exposure assessment goals. They recommend that international study guidelines should be prepared to support the production of data.

30. A human case study was presented to risk assess two mycotoxin mixtures which can occur and co-occur in cereal based food products (DON, FBs and ZEN, and T2/HT-2 toxin, DON and NIV) using the component-based approach (CBA)[6] and provisional daily intake modelling methodologies (further detailed in the Exposure assessment section). A problem formulation, exposure assessment, hazard assessment and risk characterisation were completed. A Margin of Exposure (MOE)[7] value of 100 was chosen as a reference/cut off since the considered mycotoxins were neither genotoxic nor carcinogenic. In brief, due to data gaps and limitations a robust risk assessment could not be performed, however, results from the CBA modelling approach calculated MOE values <100 for both mixtures, indicating either the need to refine the risk assessment or a potential health risk.

31. A hierarchy map (based on EU Member states) was made possible when considering exposure to T2/HT-2 toxin, DON and NIV for adults. The maps provided a visual representation of higher risk exposure groups. With reference to the co-occurrence and occurrence data collected for the United Kingdom (UK); T2/HT-2 toxin, DON and NIV adult exposure levels based on the mean co-occurrence data and mean consumption value were >0.8 ,0.6-1.2 and 0.24-0.3 µg/kg bw/day, respectively.

32. To put into context the above exposure values, their health-based guidance values (HBGV) are provided. The acute reference dose (ARfD) for T2/HT-2 toxin is 0.3 µg/kg bw (EFSA, 2017a). DON and its acetylated forms have an ARfD of 8 µg/kg bw or a tolerable daily intake (TDI) of 1 µg/kg bw (EFSA, 2017b). Whilst, NIV has a TDI of 1.2 µg/kg bw (EFSA, 2013). Therefore, the estimated exposure levels for UK adults of T2/HT-2 toxin is above the HBGV, whilst exposure levels for DON and NIV are below their respective HBGVs.

33. To conclude, the following data gaps and research recommendations were observed and/or suggested by Battilani *et al*., (2020). There is limited knowledge on the presence and co-occurrence of multiple mycotoxins, both for primary mycotoxins and their modified forms, in food and feed. Available analytical methods have limitations for the routine monitoring of modified and multi-mycotoxins in food and feed. In the context of multi-mycotoxin analysis

---

[6] Component based approaches are used to estimate the hazard or risk of combined exposures based on information on exposure and hazard for each individual component.

[7] The margin of exposure (MOE) is the ratio of the point of departure (typically the benchmark dose – lower confidence limit for a tumourigenic response in experimental animals), to the estimated human exposure for a genotoxic carcinogen. MOE values that are ≥10,000 have been considered to indicate low concern.

and the use of LC-MS methodologies; there remains an urgent need for the following: lower costs, fit for purpose methods characterised by the ability to measure multiple mycotoxins (with lower limits of quantification for all co-occurring mycotoxins; including their metabolites investigated *in vivo*), and availability of commercial reference materials for providing reliable quantitative results.

34. In terms of toxicity data, a limited number of articles on mixtures were observed in comparison to those only exploring effects of single compounds. The available studies only cover a very limited combination of mycotoxins and the available toxicokinetic data is mainly in livestock species (*e.g.* pigs and chickens), as well as rats. The modelling of TD features of mycotoxin mixtures could not be performed based on the limited number of data available. The development of prioritisation criteria for mycotoxin mixtures to be tested was suggested as a further research priority. In addition to this, consistent methodologies and harmonised guidelines for generating *in vitro* and *in vivo* TK and TD data are needed to provide consistent data for pharmacologically based toxicokinetics and benchmark dose modelling of mycotoxin mixtures. Current analytical methods should have the capability to detect and analyse real world samples. Finally, the utilisation of TK and TD modelling should be further considered and explored.

35. With reference to the use of biomarkers for exposure assessment purposes; there is a need to derive qualitative and quantitative correlations between the mycotoxin intake from food and from other possible routes of exposure like dermal or inhalation (which may be important to consider as part of occupational hazard assessments; however, it is unclear how realistic it would be to achieve this level of granularity from such a population based study).

Opinions by authoritative groups on some mycotoxin combinations

36. Assessments of some binary mycotoxin combinations have been carried out by EFSA, Joint Food and Agriculture Organization and World Health Organisation Expert Committee on Food Additives (JECFA) and the Scientific Committee on Food (SCF). These are summarised in the next following paragraphs.

EFSA reviews

37. During their review of 4,15-DAS in 2018, the EFSA Panel on Contaminants in the Food Chain (CONTAM) considered the combined effects and interactions of 4,15-DAS with T-2 and HT-2 toxins, AFs, OTA and FBs. Following the analysis of the available database describing possible effects of combined exposure to 4,15-DAS and other mycotoxins, the EFSA CONTAM Panel concluded that “the data was weak and inconclusive” (EFSA, 2018).

38. The EFSA CONTAM Panel also examined available publications addressing interactions of CIT with PAT, AFs and OTA, particularly on the subject of synergism in 2012. It was concluded by the EFSA CONTAM Panel

that "the available evidence indicated that CIT at low doses does not exacerbate the toxic effects of other mycotoxins and that the combined effect of CIT and OTA is, at most, additive" (EFSA, 2012a).

JECFA reviews

39. The JECFA first reviewed the toxicology associated with concurrent exposure to FBs and other mycotoxin agents in 2011. The reviewed *in vitro* and *in vivo* studies were found to "show inconclusive and sometimes contradictory results. The effects of simultaneous exposure tend to be at most additive. In some *in vitro* and *in vivo* studies, the authors suggested that synergism or antagonism may occur, but often only single doses of each individual mycotoxins were used", and as such the JECFA Committee concluded "that these study designs were inadequate to detect synergism", and "overall, the available data on co-exposure were inadequate for their use in its evaluation" (JECFA, 2011).

40. Although studies by Carlson *et al*., (2001) and Gelderbloom *et al*., (2002) were noted; as these documented the ability of FB1 to promote AFB1 hepatocarcinogenicity in trout and orally dosed pure FB1 in rats induced precancerous lesions, respectively. The treatment regime and dose at which these effects were observed in trout was through exposure of 100 ppb of AFB1 and then exposure to ≥ 23 FB1 ppm for 42 weeks (Carlson *et al*., 2001). Whilst in male Fisher rats (n = 5-8/group) it was AFB1 at 17 µg/kg bw per day (*via* oral gavage) for 14 days, and exposure to FB1 at 250 mg/kg diet (post recovery period of 21 days in between) (Gelderbloom *et al*., 2002). The JECFA Committee noted that "co-exposures to AFB1, a compound with known genotoxic properties, and FBs, which have the potential I to induce regenerative proliferation, would of concern" (JECFA, 2011).

41. The topic was revisited in the JECFA 2018 evaluation (Riley *et al*., 2018). Synergistic effects were reported by Qian *et al*., (2016) for the development of preneoplastic lesions (*e.g.* increased number of apoptotic cells and placental form of glutathione-*S*-transferase), where male F344 rats (n=13) were exposed to pure AFB1 (equivalent to 15 µg/kg bw per day for 14 days) and pure FB1 (equivalent to 25 mg/kg bw per day for 21 days), alone or sequentially (the rats were treated with AFB1 and then FB1, with a recovery period of 21 days in between).

42. JECFA concluded that even though there are additive or synergistic effects observed from FB1 and AFB1 co-exposure in laboratory animals in inducing the development of preneoplastic lesions and hepatocellular carcinoma (as discussed above), there was currently no data available on such effects in humans. Furthermore, two prospective epidemiological studies (Magoha *et al*., 2016; Shirima *et al*., 2015), do not support the hypothesis of an interaction between AFB1 and FB1 in childhood stunting. JECFA concluded that there were few data available to support co-exposure as a contributory factor in human disease. However, the interaction between AFB1 (genotoxic), and FBs, which have the potential to induce regenerative cell

proliferation (particularly at exposures above the provisional maximum tolerable daily intake), remained a concern.

43. It was recommended that exposures to both compounds (*i.e.* FB1 and AFB1) should be reduced and that emphasis on human studies should be on biomarker-based approaches.

44. JECFA further assessed the combined toxicity of pure FBs and pure DON in Swiss mice (Kouadio *et al*., 2013) in their 2018 evaluation. The JECFA Committee concluded that "co-exposure suggested additivity, and the effects on growth, clinical chemistry and biochemical parameters were possibly more than additive… the reduced weight gain was seen at a very low dose of AFB1 compared to other studies" (JECFA, 2018).

SCF review

45. In 2002, the SCF -published an opinion to evaluate whether the establishment of a group TDI for four trichothecenes (T-2 toxin, HT-2 toxin, DON and NIV) was appropriate and if so, the feasibility of performing it (SCF, 2002).

46. The SCF were in favour of establishing a group TDI for several compounds when they shared a common mode of action and there was frequent co-exposure. At the time of review, there were few studies found that addressed the effects of combined exposure to several trichothecenes.

47. Although different types of trichothecenes appear to cause similar toxic effects at the biochemical (strong inhibitory effect on the protein synthesis by binding to ribosomes, inhibitory effect on RNA and DNA synthesis) and cellular level(toxic effect on cell membranes), the SCF noted that "it is not clear whether toxins work *via* identical mechanisms at the biochemical and cellular level." Additionally, "there are also considerable differences in the spectrum of toxic effects *in vivo*. Large, non-systematic potency differences between these toxins were seen when different endpoints are being considered".

48. In *in vitro* studies, dose additivity as well as antagonism has been observed for T-2 toxin, DON and NIV, whilst in *in vivo* studies only antagonism was observed for the combination of T-2 toxin and DON, and no dose additivity was observed. At the time of review, the SCF were not aware of any other combinations of NIV with other trichothecenes examined *in vivo*. As such with only *in vitro* studies suggesting dose additivity, the establishment of the nature of combined effects or relative potencies of trichothecenes was not further explored. In conjunction to this, the SCF considers "the available data, while limited, did not support the establishment of a group TDI for all trichothecenes evaluated, as synergism was not observed".

MYCOMIX

49. The MYCOMIX project (funded by the Portuguese Foundation for Science and Technology) carried out in 2013-2015 aimed to contribute and fill the gap concerning the risk assessment of children to multiple mycotoxins in infant foods (Alvito *et al*., 2015). Three questions were postulated. Firstly, are children exposed daily to one or several mycotoxins *via* the diet. Secondly, can this co-exposure affect children's health, and lastly are there interactive effects in toxicity of mixtures of mycotoxins.

50. An overview of the risk assessment under the MYCOMIX project was published in 2018 (Assunçao *et al*., 2018). Analysis of 52 different cereal-based products revealed a co-occurrence of mycotoxins in 75% of the analysed samples, with two or more mycotoxins occurring simultaneously. The highest number of mycotoxins detected simultaneously was seven and the combinations of two (OTA and DON; OTA and FBs) and four (AFs, OTA and ZEN) mycotoxins were the most commonly detected, with a percentage of occurrence of 6% for each combination. Note that all analysed samples had mycotoxin levels below the limits specified in the legislation (Regulation (EC) No 1881/2006)[8].

51. Food diary analysis revealed that ~92% of the children (n=75; 18 males and 20 females (13-24 months), 9 males and 9 females (25-36 months), 7 males and 12 females (36-47 months)) consumed one or more cereal-based products, and at least once in three days. 42%, 65% and 65% consumed breakfast cereals, infant cereals and biscuits, respectively. The mean daily consumption of these food groups, for all children (both non-consumers and consumers), were 5.6 g (breakfast cereals), 25.3 g (infant cereals) and 8.7 g (biscuits). For the only consumers group, the values increase to 15.4 g, 38.7 g, and 13.4 g, for the same food groups respectively.

52. Worst-case exposure for the summed daily intake of mycotoxins present in cereal-based products (breakfast cereal, infant cereal and biscuits) are presented in *Table 2*.

53. Population-based exposure includes the estimation of intake for all respondents (including non-consumers) and allows the identification of food groups that have made the highest contribution to overall intakes. Breakfast cereals were the highest contributor for the estimated daily intake of mycotoxins by Portuguese children under three years old, revealing the highest values for FBs, trichothecenes, ZEN and AFB1. On the other hand, processed cereal-based foods (flours) presented the highest contribution for the estimated daily intakes of AFM1, AFB2, AFG1, and OTA.

[8] Regulation (EC) No 1881/2006 concerning setting maximum levels for certain contaminants in foodstuffs, which is available on the EUR-Lex website.

Table 2 - Sum of worst-case children's daily intake of mycotoxins present in cereal-based products (breakfast cereal, infant cereal and biscuits) based on a deterministic approach (reproduced from Assunçao *et al*., 2018).

	Consumers and non-consumers	**Only consumers**
Toxins	**Sum of daily intake (ng/kg bw/day)**	
AFM_1	0.069	0.116
AFB_1	0.012	0.028
AFB_2	0.003	0.006
AFG_1	0.016	0.028
OTA	0.131	0.227
FB1	6.4	14.0
FB2	1.0	2.6
DON	57.22	112.78
NIV	2.68	6.60
ZEN	0.86	1.64

Abbreviations: AFM_1; Aflatoxin M1, AFB_1; Aflatoxin B1, AFB_2; Aflatoxin B2, AFG_1; Aflatoxin G1, OTA; Ochratoxin A, FB_1; Fumonisin B1, FB_2; Fumonisin B2, DON; Deoxynivalenol, NIV; Nivalenol, ZEN; Zearalenone.

54. The risk characterisation for AFs was determined by MOE calculations, whilst for the remaining mycotoxins hazard exposure quotients (HI; individual mycotoxins) and hazard exposure indexes[9] (HQ; combined mycotoxins) were used. MOE values for AFB_1 and AFG_1 for the 90th, 95th and 99th percentiles were below 10,000 suggesting a potential health concern. The highest HQ and HI was for DON and the simultaneous exposure to trichothecenes, respectively. Although, these values were <1 indicating no cause for concern.

55. The authors note that results should take into account the identified uncertainties including; the number of analysed samples, the number of children in the cohort, and the available toxicological data. Additionally, the exposure was assessed using an indirect approach (*i.e.* combining data of mycotoxin occurrence in food and food consumption which has its own associated limitations[10]).

56. From the obtained results, the authors suggest that actions should be set in order to protect this population group recommending a national monitoring program to be carried out with the aim of establishing protective values in legislation. Additionally, further research should be conducted to

[9] A hazard exposure index is a risk-assessment tool, which can indicate whether further investigations are required for mixtures. It is based on dose addition assumptions; it is the sum of the hazard quotients of the chemicals in the mixture.
[10] These limitations include the assumption of heterogenous distribution of mycotoxins in food, exposure from other routes of exposure excluding oral ingestion, the potential presence of masked mycotoxins, the influence of food processing, inter-individual variation in toxicokinetic profiles, and the under- and overestimation of food consumption data.

obtain toxicological data, including health consequences resulting from early-life exposures to multiple mycotoxins. Good farming and food production processes should aim to reduce the generation of mycotoxins in crops, and greater consideration of decontamination of foods destined for children consumption.

MYTOX

57. MYTOX[11] is a multi-disciplinary research group, which deals with issues involving toxigenic moulds, mycotoxins, mycotoxins and human health, and mycotoxins and animal health.

58. The mycotoxins and human health research unit includes all research projects in relation to the occurrence of mycotoxins and its effects on human health including epidemiological studies, risk assessment studies and scenario analyses. The Secretariat has confirmed with the MYTOX coordinator (De Saeger, personal communication, 2021) that the research group has several ongoing/or about to start projects to evaluate the impact of multi-mycotoxin exposure, and that publications are expected in the future.

Toxicokinetics

59. The Committee previously reviewed the available toxicokinetic data relating to combined exposures to mycotoxins (Warth *et al*., 2013; Battilani *et al*., 2020), and concluded that there is a limited number of studies in order to fully understand the toxicokinetic profiles of varied mycotoxin mixtures *in vivo* in humans.

60. Battilani *et al*., (2020) further highlighted the complexity of studying the toxicokinetic of mycotoxins mixtures, suggesting that it needs to be addressed on a case-by-case approach. Mycotoxin dosage, exposure pathway, interspecies and intraspecies differences were identified among the most important parameters that may influence the toxicokinetics of mixtures.

Toxicology

61. This section is presented in three parts. Firstly, the Committee's review on the toxicity of single mycotoxins. Secondly, the review of the available data on relative potencies for mycotoxin groups, and lastly, the review of the toxicity of common binary mixtures found in literature.

62. The Committee has previously reviewed the toxicity of single mycotoxins; especially in the diet of infants and children aged 0-12 months

[11] Further information can be found at the MYTOX website.

and 1-5 years, respectively (TOX/2017/30)[12]. Annex B of TOX/2020/44[13], presented an overview of all mycotoxins previously covered in the scope of TOX/2017/30. The collated information includes; their associated mycotoxins, the species of fungus that produces them, their mode of action (MOA), key toxicological endpoints, as well as their recommended HBGVs as set by authoritative bodies such as EFSA, JECFA, SCF *etc.*

63. In mixtures toxicology, there are three main different categories of interactions (SCHER, SCCS, SCENIHR, 2012; EFSA, 2019) between mycotoxins. These are:

i). Similar action (dose/concentration addition) occurs if chemicals in a mixture act by the same mechanism/MOA, and only differ in their potencies;
ii). Dissimilar action (independent action) occurs if chemicals act independently from each other, usually through different MOA that do not influence each other and;
iii). Interactions describes the combined effect of two or more chemicals as stronger (synergistic, potentiating, supra-additive) –or weaker (antagonistic, inhibitive, sub-additive, infra-additive) than would be expected on the basis of dose/concentration addition or response addition. The observed interactions are therefore dependent to the relative dose levels, the exposure route(s), timing and duration of exposure, and the biological target(s).

64. A paper by Speijers & Speijers (2004) on combined toxic effects was identified to be one of the first reviews to assess the topic. It concluded that, at the time, tools were not fully developed to establish the type of interaction or whether there is any interaction at all (with regards to trichothecenes). More recent reviews have also been published by Grenier and Oswald (2011), De Ruyck *et al*., (2015), Alassane-Kpembi *et al*., (2017), Lee & Ryu (2017), and Battilani *et al*., (2020).

65. Authoritative groups such as EFSA, JECFA, and SCF have provided opinions on some binary mycotoxin mixtures (refer to paragraphs 19-48).

66. The COT reviewed literature on binary mixtures as the co-occurrence of two mycotoxins in food commodities is more commonly reported. A summary of OTA, AFB1 and *Fusarium* spp. mycotoxins and their interactions with other mycotoxins is presented in *Tables 3-5*, respectively. These experiments were conducted for a range of different durations and concentrations (as detailed in TOX/2020/34[14]), it was not possible to draw specific conclusions on these and so the details are not included in this statement.

[12] The first scoping paper TOX/2017/30 and the resulting addendum to the 0-5 years Overarching Statement are available on the COT website.
[13] Annex B of TOX/2020/44 is available on the COT website.
[14] TOX/2020/34 is available on the COT website.

67. The majority of studies testing for the combined effects of mycotoxins are *in vitro*, with cell viability endpoints (*e.g*. apoptosis, necrosis, DNA damage, oxidative damage and immunotoxicity) being the most commonly assessed. Available *in vivo* data reported potential adverse effects on the liver, kidneys and teratogenicity.

68. Other considerations for the potential adverse effects on the microbiota (Baines *et al*., 2013; Liew & Mohf-Redzwan, 2018) and endocrine system (Demaegdt *et al*., 2016) were also reviewed by the COT.

69. The COT observed that there were a number of mycotoxins with MOAs involving ribosomal protein synthesis inhibition; however, there was a lack of information on possible additive toxicity. Additionally, there is a large amount of variability in the methodology utilised since there is currently no harmonisation on combinative testing strategies for each toxicological endpoint for each plausible mycotoxin combination.

Table 3 - Provides a highlight summary of the observed combinative effects of ochratoxin A with fumonisin B1, zearalenone, and citrinin in different *in vitro* methods.

Mycotoxin mixture	Method	Cellular or animal model	Endpoint	Combination effect	Reference
OTA + FB_1	*In vitro*	Rat C6 glioma, Vero monkey and human Caco-2 cells	Cytotoxicity	Synergistic	Creppy *et al*., (2004)
	In vitro	PK-15 cells	Cytotoxicity	Additive	Šegvić Klarić *et al*., (2007)
	In vitro	human and pig lymphocytes	Cytotoxicity	Synergistic	Mwanza *et al*., (2009)
	In vitro	Male Wistar rats	Genotoxicity	Synergistic	Domijan *et al*., (2006)
OTA + ZEN	*In vitro*	hHepG2 cells	Cytotoxicity	Antagonistic	Wang *et al*., (2014)
	In vitro	hHepG2 and KK-1 cells	Cytotoxicity	Additive	Li *et al*., (2014)
OTA + CIT	*In vitro*	Piglets lymphocytes	Immunotoxicity	Synergistic	Bernhoft *et al*., (2004)
	In vitro	Monkey kidney vero cells	Cytotoxicity	Synergistic	Bouslimi *et al*., (2008b)
	In vitro	PK-15 cells	Cytotoxicity	Antagonistic	Šegvić Klarić *et al*., (2012)

Abbreviations: OTA = Ochratoxin A; FB1 = Fumonisin B1; ZEN = Zearalenone; CIT = Citrinin; PK-15 = Porcine kidney 15 epithelial cells; hHepG2 = human hepatoma cells G2; KK-1 = murine ovarian granular cells.

Table 4 - Provides a highlight summary of the observed combinative effects of aflatoxin B_1 with aflatoxin B_2, fumonisin B_1, deoxynivalenol, zearalenone, T-2 toxin, and ochratoxin A in different *in vitro* and *in vivo* models.

Mycotoxin mixture	**Method**	**Cellular or animal model**	**Toxicity endpoint**	**Combination effect**	**Reference**
AFB_1 + AFB_2	*In vitro*	hUVEC	Cytotoxicity	Synergistic	Braicu *et* al., (2010)
	In vitro	hLF and A 2780	Cytotoxicity	Additive	
	In vitro	Rat liver slices	Toxicity	Antagonistic	Friedman *et al.*, (1997)
AFB_1 + FB_1	*In vitro*	Spleen mononuclear cells	Cytotoxicity	Synergistic	Mary *et al.*, (2012)
	In vitro	hHep-G2; hBEAS-2B cells	Cytotoxicity	Antagonistic; Additive	McKean *et al.*, (2006a)
	In vivo	Fischer 344 rats	Acute	Synergistic	
	In vivo	Male Fischer rats	Hepatotoxicity	Synergistic	Gelderblom *et al.*, (2002)
	In vivo	White rabbits	Hepatotoxicity	Synergistic	Orsi *et al.*, (2007)
	In vivo	Male Wistar rats	Hepatotoxicity	Synergistic	Theumer *et al.*, (2008)
	In vivo	Maler F344 rats	Hepatotoxicity	Synergistic	Qian *et al.*, (2016)
AFB_1 + DON	*In vitro*	PK-15 cells	Cytotoxicity	Synergistic	Lei *et al.*, (2013)
	In vitro	BRL 3A cells	Cytotoxicity	Synergistic	Sun *et al.*, (2015)
	In vitro	Ames test	Mutagenicity	Synergistic	Šmerák *et al.*, (2001)
AFB_1 + ZEN	*In vitro*	PK-15 cells	Cytotoxicity	Synergistic	Lei *et al.*, (2013*)*
	In vitro	BRL 3A cells	Cytotoxicity	Synergistic	Sun *et al.*, (2015)
AFB_1 + T-2	*In vitro*	Ames test	Mutagenicity	Synergistic	Šmerák *et al.*, (2001)
	In vitro	hBEAS-2B cells	Cytotoxicity	Additive and synergistic	McKean *et al.*, (2006b)
	In vivo	Fischer 344 rats	Acute	Additive	
AFB_1 + OTA	*In vitro*	Ames test	Mutagenicity	Synergistic	Sedmíková *et al.*, (2001)
	In vitro	Monkey kidney vero cells	Cyto and genotoxicity	Additive	Golli-Bennour *et al.*, (2010)
	In vitro	hHep-G2 cells	Cytotoxicity	Additive	Corcuera *et al.*, (2011)
	In vitro	hHep-G2 cells	Genotoxicity	Antagonistic	
	In vivo	Wistar rat dams	Teratogenicity	Antagonistic	Wangikar *et al.,* (2004)
	In vivo	Male Sprague Dawley rats	Hepatotoxicity and nephrotoxicity	Synergistic	Abdel-Wahhab *et al.*, (2015)

Abbreviations: AFB_1 = Aflatoxin B_1; AFB_2 = Aflatoxin B_2; FB1 = Fumonisin B_1; DON = Deoxynivalenol; ZEN = Zearalenone; T-2 = T-2 toxin; OTA = Ochratoxin A; HUVEC = Human umbilical vein endothelial cells; HLF= Human lung fibroblasts; hHep-G2 = Human hepatoma G2 cells; hBEAS-2B = Human bronchial epithelial cells; PK-15 = Porcine kidney 15 epithelial cells; BRL 3A = Buffalo rat liver cells.

Table 5 - Provides a highlight summary of the observed combinative effects of various combined *Fusarium* mycotoxins including: zearalenone and fumonisin B1; zearalenone and T-2 toxin; deoxynivalenol and zearalenone; deoxynivalenol and T-2 toxin; deoxynivalenol and 15-acetlydeoxynivalenol; deoxynivalenol and nivalenol; and deoxynivalenol and fumonisin B_1 in different *in vitro* and *in vivo* methods.

Mycotoxin mixture	Method	Cellular or animal model	Endpoint	Combination effect	Reference
ZEN + FB_1	*In vitro*	Human Caco-2 cells	Cytotoxicity	Antagonistic	Kouadio *et al*., (2007)
			Lipid peroxidation	Synergistic	
			Inhibition of DNA synthesis	Antagonistic	
			DNA fragmentation	Synergistic	
ZEN + T-2	*In vitro*	hCFU-GM cells	Myelotoxicity	Additive	Ficheux *et al*., (2012)
DON + ZEN	*In vitro*	hCFU-GM cells	Myelotoxicity	Additive	
	In vitro	HCT116 cells	Cytotoxicity	Antagonistic	Bensassi *et al*., (2014)
DON + T-2	*In vitro*	hCFU-GM cells	Myelotoxicity	Additive or synergistic	Ficheux *et al*., (2012)
DON + 15-AcDON	*In vitro*	hGES-1 cells	Cytotoxicity	Synergistic	Yang *et al*., (2017)
DON + NIV	*In vitro*	Human Caco-2 cells	Cytotoxicity	Synergistic and additive	Alassane-Kpembi *et al*., (2013)
	In vitro	hGES-1 cells	Cytotoxicity	Synergistic	Yang *et al*., (2017)
DON + FB_1	*In vitro*	Human Caco-2 cells	Cytotoxicity	Synergistic	Kouadio *et al*., (2007)
	In vitro	hCFU-GM cells	Myelotoxicity	Antagonistic	Ficheux *et al*., (2012)
	In vivo	Male crossbred castrated piglets	Morphological changes	Antagonistic	Bracarense *et al*., (2012)
			Immunological changes	Synergistic-Antagonistic	

Abbreviations: ZEN = Zearalenone; T-2 = T-2 toxin; DON = Deoxynivalenol; 15-AcDON = 15-acetyldeoxynivalenol; NIV = Nivalenol; FB_1 = Fumonisin B_1; hCFU-GM = Human colony forming unit-granulocyte and macrophage cells; HCT116 = human colon carcinoma cell line; GES-1 = Human gastric epithelial cells.

Exposure assessment

70. As stated previously, the co-occurrence of mycotoxins in food and feed is possible since some fungi species are able to produce more than one mycotoxin (for example the *Fusarium* spp.), food commodities can also be contaminated by several fungi species. A combined exposure is therefore highly likely in humans due to varied diets and/or one food commodity may be contaminated with more than one mycotoxin.

71. The completion of an exposure assessment is challenging when limited information is available. Little to no UK relevant data could be obtained from the literature where all age groups were considered. It would be necessary to assess all age groups to determine those who would be of greater risk. Additionally, different methodologies have been observed in the literature to assess the levels of exposure (*e.g*. food diaries, biomarker analyses etc), as such performing data comparison may not be accurate. The development and application of multi-analyte methods has been advancing as detailed earlier; however, this has not yet been internationally applied as a gold standard for assessing the presence of multiple mycotoxins in food commodities. The use of current methodologies for mycotoxin analysis (e.g. HPLC) still presents an issue in terms of management of left-censored data.

Stepwise approach

72. A stepwise approach to the exposure assessment was considered by the Committee (summarised below).

73. Firstly, mycotoxins should be categorised based on toxicological similarities where an endpoint is defined. This will then determine how occurrence data for the considered mycotoxins should be grouped together to calculate total residues for each mycotoxin group by summation in the exposure assessment (either in one food or multiple foods). An opportunity to note any missing data can be recorded throughout this step.

74. The exposure should be then calculated deterministically, and if major exceedances are observed in relation to the toxicological endpoint a probabilistic calculation should be considered. The estimated exposure can then be compared against the health-based guidance value to determine the MOE. Depending on the endpoint, an MOE value that is ≤100 (for non-genotoxic and non-carcinogenic compounds) or 10,000 (for genotoxic carcinogens) would indicate a level of risk whilst values that are ≥100 or 10,000 indicates no appreciable cause of concern (EFSA, 2012b). The MOE value aids in putting exceedances into perspective.

75. Lastly, if probabilistic modelling was carried out; a sensitivity analysis should be considered for assessing the impact of different variables.

Potential data sources

76. Three potential data sources to use in the exposure assessment were proposed and summarised in the following paragraphs.

FSA – Mycotoxin Total Diet Study

77. The FSA has previously carried out a Total Diet Study (TDS)[15] that included mycotoxin analysis in 2011 (FS102081), where a total number of 3, 312 food samples were analysed for the presence of mycotoxins. The main aim of the study was to calculate background exposure to various mycotoxins from the whole diet and to compare exposure to those calculated by other sources (Stratton *et al*., 2017).

78. Co-occurrences were observed in the TDS dataset. For example, sample S14-042859 (a wholemeal bread) contained DON, some ergot alkaloids and also a low level of OTA.

79. The following limitations were observed with the TDS dataset including; that is was limited to a small number of food groups, some recovery rates were poor, food samples were collected from 2009 and as such may not be reflective of the current levels of mycotoxins detected in foods. Most results were at or below the limit of quantification (LOQ), meaning that much of the data is left-censored as the values could not be accurately determined. Therefore, the lower- and upper- bound estimates were derived from the occurrence data to reflect the uncertainty in the exposure assessment. This approach has been discussed by EFSA in their Scientific Report on the Management of left-censored data in dietary exposure assessment of chemical substances (EFSA, 2010). Here, EFSA comment that the robustness of this approach is limited by the computational difficulties of calculating percentiles and basic statistics, if the percentage of left censorship is large and in the application of statistical techniques like regression methods (EFSA, 2010). Finally, as mentioned multi-mycotoxin analysis was not consistently used for each food sample.

80. Further information received from the project manager (MacDonald, personal communication, 2020) has confirmed that a method for multi-mycotoxin analysis (*i.e.* different classes/families) was not performed for the TDS. This was due to the different chemistries and properties of the mycotoxins themselves rather than the different food matrices. Therefore, samples were analysed by several methods to obtain the full suite of analyte results with the lowest reporting limits achievable. Although not strictly a multi-analyte method, mycotoxins from the same family (*e.g*. ergot alkaloids and trichothecenes) were detected using one methodology. The possibility and availability of a multi-mycotoxin method were also discussed with the project manager. It was confirmed that a methodology is both possible and available although this means that compromises have to be made in order to make it

[15] The Food Standards Agency Total Diet Study on Mycotoxins can be found on the FSA website.

suitable for all tested mycotoxins. The compromises include the lack of dedicated extraction techniques, sample clean-up and analyte enrichment. This results in higher reporting limits, which in turn can affect the estimate of intake (*i.e*. overestimation), as well as potentially requiring additional resources in terms of sample re-analyses to avoid false positives.

EFSA – MYCHIF Platform

81. It was noted that UK co-occurrence data was presented in the scientific report for MYCHIF, therefore the MYCHIF platform (Battilani *et al*., 2020) was included as a potential data source.

82. A human case study was presented as part of the MYCHIF report (Chapter 3.8.2, pp. 88) where an aggregate chronic exposure assessment for two mycotoxin mixtures in cereal food sources (1: DON, FBs and ZEN and 2: T-2/HT-2 toxin, DON and NIV) was carried out using two modelling methodologies: CBA and provisional daily intake approach.

83. In the CBA, the co-occurrence of mycotoxins and consumption data of cereal-food based products (as an example) for each single mycotoxin were combined to obtain an individual mycotoxin exposure, this was then summed up to obtain the total exposure, under the dose addition assumption. The main uncertainty identified was the adopted deterministic approach of the input modelling data for mycotoxin concentrations. Due to the scarcity of concentration data for many countries, a probabilistic approach was applied at an EU level only. It was assumed that the maximum exposure limits (*i.e.* the lower and upper bound highest 95th percentile chronic exposure) were the most conservative values. The risk decision was based on the calculated MOE values, based on the methodology and assumptions in the MYCHIF case study the MOE values were <100 for all age groups (adolescent, adult and elderly).

84. The provisional daily intake (expressed in µg/kg bw/day) models the internal dose with the available human biomarker data to derive exposure to the mixture. This was estimated by combining the mycotoxin concentration in the urine, the available excretion rate for each of the mycotoxin in the mixture, the human body weight and the daily urine excretion volume (µg/L mycotoxin, L urine in 24 hrs, % excretion rate, kg bw, respectively). Values were calculated for single mycotoxins present in the mixture and for the mixture. A hazard exposure index was used to estimate the risk, if the value is ≤1 the combined risk as deemed acceptable, whereas when it is >1 a potential concern is possible. The identified uncertainties included the default body weight of 70 kg, the excretion rate where values were derived from a single study or from correlation approximations, urine volumes were the urine was not corrected for dilution factors, and data representativeness. Overall, a hazard exposure index could not be quantified due to the uncertainties for single mycotoxins described above, since these would also need to be integrated into the analysis of mixtures were additional variables should be considered for unknown toxicokinetics and toxicodynamics, as well as unknown synergistic/additive effects.

85. The MYCHIF data was located and the file downloaded. Extraction of relevant UK data was attempted; however, the datasets are extremely complex and were not accompanied with straightforward guidance. The Secretariat has made the relevant contact in order to gain further guidance. The following benefits to using this data have been observed; the use of multi-mycotoxin analysis, the more recent data collection of mycotoxin co-occurrences in cereal commodities, and the integration of singular and multi-biomarker mycotoxin analysis data.

86. Nevertheless, at present it has been difficult to determine potential limitations due to the complexity of the datasets. Uncertainties for both modelling methodologies were also identified.

Exposure data derived from the literature

Non-UK data

87. Co-exposures have been reported in the literature for various age groups such as those observed from the MYCOMIX Portuguese studies in children as seen in paragraph 47.

88. For the exposure assessment, non-UK co-occurrence data and UK consumption data for each single mycotoxin could be used to obtain an individual mycotoxin exposure. These could then be totalled for each co-occurrence type and also for each mycotoxin family to obtain the total exposure within each food or food group.

89. Obvious limitations include the use of non-UK data which may not be applicable to consumers in the UK, however, the use of non-UK data may expedite an exposure assessment to reveal common mycotoxin combinations as well as the most affected food groups in Europe.

Human biomonitoring data

90. The use of human biomonitoring (HBM) data was explored as part of TOX/2020/44[16], outputs from this exercise are summarised below.

91. Biological monitoring utilises biomarkers[17] to represent or estimate the internal exposure as a result of inhalation, ingestion or dermal exposure to a chemical, and as such, biomarkers are indicators of exposure, effect, and/or susceptibility. Exposure assessments to any dietary contaminant is based on intakes from food (or feed), otherwise known as the external exposure or oral

[16] TOX/2020/44 is available on the COT website.
[17] A biomarker is a naturally occurring molecule, gene, or characteristic by which a particular pathophysiological or physical process, disease etc. can be identified.

dose. However, the bioaccessibility[18] and bioavailability[19] of the contaminant determines the internal exposure.

92. Mycotoxins can be classified as short-lived chemicals that can only be effectively measured if the individual is undergoing continuous or continual exposures or if the timing of exposure(s) is known. Mycotoxin biomarkers have been defined as the compounds themselves (*e.g*. parent compounds and/or a metabolite) or as a result of interaction with target molecules (e.g. DNA or protein adducts) (Marín *et al*., 2018). Urinary excretion mainly represents recent mycotoxin intake, whereas measurements in plasma/serum are more likely to represent long-term exposure.

93. The main analytical methods employed to perform biomarker analyses are based on either chromatography (*e.g.* LC) or immunochemistry (*e.g.* ELISA).

94. European human biomonitoring initiatives such as the Consortium to Perform Human Biomonitoring on a European Scale (COPHES)[20], Human Early-Life Exposome (HELIX)[21], and European Union Human Biomonitoring (HBM4U)[22], as well as typical literature databases (PubMed, Science Direct, Google Scholar, Scopus and Zenodo) and the Information Platform for Chemical Monitoring (IPCheM) platform – were mined for any relevant UK biomonitoring data on combined exposures to mycotoxins.

95. Both COPHES and HELIX did not include exposure to mycotoxins in the scope of their work, however, in the HBM4EU initiative it was. In brief, Alvito *et al*., (2019)[23] conclude that there are numerous factors that need to be considered when attempting to integrate biomarker data for exposure assessment – and thus the following risk assessment. These factors include: the validation and harmonisation of analytical methods to assess mycotoxin exposure biomarkers, a greater understanding of the current exposure levels of the European population to multiple mycotoxins and whether this differs for each Member State *etc*.

96. It was found that there are no UK government led HBM initiatives relating to mycotoxins, however, scientific interest for this has and continues

[18] Bioaccessibility describes events that take place during food digestion for transformation into potentially bio-accessible material, the absorption/assimilation through epithelial tissue and pre-systemic metabolism.
[19] Bioavailability describes the fraction of bio-accessible material which is likely to reach the systemic circulation.
[20] The COPHES final report is available on the EU HBM website, a brief technical report is also available.
[21] Further information on the HELIX project is available on the CORDIS EUROPA website.
[22] A pdf file for a brief informative guide for HBM4EU is available on the HBM4EU website.
[23] The HBM4EU mycotoxin paper by Alvito *et al*., (2019) is available on the HBM4EU website.

to grow which has led to several publications. These publications were previously reviewed by the COT. It was observed that the available literature seems to focus on estimating DON exposures from using total DON (free DON and DON-glucuronides) in urinary samples as biomarkers in the UK population. Only one other study for OTA exposure was reported by Gilbert *et al*., (2001).

97. The presence of DOM-1, a metabolite that is formed by microbiota metabolism in urine is rarely reported suggesting that its it may not be a suitable as an exposure biomarker for DON (Turner *et al*., 2008a, 2008b, 2009, 2010, 2011; Hepworth *et al*., 2012; Wells *et al*., 2016; Papageorgiou *et al*., 2018a, 2018b). From the reviewed studies, a proportion of the UK population (adults, children, adolescents, pregnant women, elderly and vegetarians) exceeded the TDI for DON of 1 µg/kg bw (EFSA, 2017b).

98. At the time of review, only one report by Gratz *et al*., (2020) performed multi-mycotoxin biomarker analyses. In this pilot study, UK children (n=21) were estimated to frequently exceed the TDI for 52% of DON and 95% of OTA cases.

Summary of exposure assessment

99. As discussed, there is little to no relevant UK co-occurrence data for mycotoxins. Available data either from food surveys, total diet studies and other databases have their own associated limitations.

100. Although the advancement and availability of detection techniques and equipment has progressed the development of biomarkers of exposure to mycotoxins are limited mainly to AFs, OTA, DON, FBs, ZEN and to a lesser extent – emerging mycotoxins such as Fus-X, CIT, NIV, T-2 toxin, 4,15-DAS, ENNs, ALT and tenuazonic acid.

101. Understanding the toxicokinetics of mycotoxin metabolites and their availability in different biological samples (*e.g*. OTA has the potential to be transferred to breastmilk) and how they may correlate to the exposure still needs further investigation. Additionally, there is a lack of harmonisation in the experimental settings and design, with particular reference to data collection and in the definition of performance criteria of fit for purpose analytical methods. Oversights on sampling strategy were also noted, for example the lack of knowledge regarding the stability of the biomarker, the defined time of sampling, and detailed information regarding the way of sample collection and storage. Furthermore, there is a need for the development of biomarkers of exposure for the detection of masked mycotoxins.

102. As such it was concluded by the COT that further research is required to enable the inclusion of HBM data for a robust exposure assessment.

Risk assessment

103. It is generally accepted that the potential effect(s) of a combination of mycotoxins may cause strong or weak interactions (as previously defined in paragraph 63.

104. The combined toxic effects that are observed will greatly depend on the experimental design. Factors such as the type of experimental cells or animal models, the duration of exposure, the dosage and relation between mycotoxins (*i.e*. the ratio of each mycotoxin in the mixture), the tested endpoint and methodology used; including any statistical aspects used for modelling scenarios.

105. Potential uncertainties could arise when comparing between toxicity studies utilising "*natural*" contaminated test samples and purified extracts. For example, in livestock studies where feed is naturally contaminated with DON, a higher toxicity was observed when compared to exposure groups treated purified DON. This result was attributed to the presence of additional fungal metabolites, where low concentrations of ZEN or the 3- or 15-AcDON precursors were found in some cases.

106. The main challenges in assessment of multiple mycotoxins include; the lack of accurate information regarding the toxicokinetic profiles of mycotoxin mixtures and their bioaccessibility (*i.e*. the actual percentage of mycotoxins that can be absorbed in the small intestine) that would enable a more accurate exposure and thus subsequent risk assessment. Additionally, the variability within mycotoxin bioaccessibility values depends on the compound, food product, contamination level and the nature of contamination (spiked or naturally contaminated). Furthermore, the breadth of *in vitro* digestion models used to assess the bioaccessibility of mycotoxins constitutes another important challenge. The suitability and reliability of *in vitro* models for *in vivo* extrapolation are also required to be comparable to the situation *in vivo*.

107. The mycotoxin absorption constitutes as another challenge considering that toxins could reach the intestine as the parent compound or as metabolites formed during digestion; the available methods for mycotoxin metabolites are also still in its infant stages. Studies on combined genotoxic effects of mycotoxin mixtures should also be further developed.

108. As for the exposure assessment, there is little to no relevant UK co-occurrence data for mycotoxins and HBM data. Available data either from food surveys, total diet studies and other databases have their own associated limitations and at this time an exposure assessment could not be performed.

109. In terms of risk assessment, one of the main challenges posed to risk characterisation is the absence of toxicological data. A deeper understanding of the interactions between multiple mycotoxins at a molecular level will assist in drawing real life conclusions on the health impact of human exposure to mycotoxin mixtures.

110. Based on the limitations discussed above and the absence of an exposure assessment, a full risk assessment on the potential adverse effects of aggregate dietary exposure to mycotoxins could not be performed by the COT.

Further considerations

111. Concerns over the effect of climate change on fungi and host specific interactions were mentioned previously in this document. Moretti *et al*., (2019) provided a narrative review on the potential emerging mycotoxin risks under a climate change scenario in Europe. It was hypothesised that the contamination risk of aflatoxin (produced by *Aspergillus flavus*) in maize in South and Central-Europe will extend to new regions in the next 30 years. The *Fusarium* spp. species profile on wheat are also hypothesised to change in Northern, Central and Southern-Europe. It is unknown whether these changes will be similar across these regions. As a result, new combinations of mycotoxins/host plants/geographical areas may arise. It was recommended by Moretti *et al*., (2019) that developments of new diagnostic tools, a deeper knowledge of both biology, and genetics of toxigenic fungi may be required.

112. Co-exposure to mycotoxins from breast milk and infant formula may also need to be considered in infants and young children. Several publications have already considered this as a potential risk. For example, Ortiz *et al*., (2018) have investigated the multiple mycotoxin exposure of infants and young children (0-23 months) *via* breastfeeding and complementary/weaning foods consumption in Ecuadorian highlands and Braun *et al*., (2020) whom performed a longitudinal assessment of mycotoxin co-exposures in exclusively breastfed infants in Austria.

Summary

113. Mycotoxins are toxic secondary metabolites produced by fungi and is capable of causing adverse health effects in both humans and animals. Those of greatest concern to human health are produced by several fungal genera of filamentous fungi, namely *Aspergillus*, *Fusarium* and *Penicillium* spp.

114. DON, FBs, and ZEN are the most prevalent mycotoxins in the world with regards to cereals and cereal based products, with a prevalence of 66%, 56%, and 53%, respectively. Regulation limits for these compounds are based on considerations for the toxicity of single exposures. There are several reports were co-exposure to multiple mycotoxins are observed in both humans and animals. As such, the literature was reviewed to investigate the potential risks of aggregate dietary exposure from mycotoxins.

115. The co-occurrence of mycotoxins in food and feed is quite common since some fungi can produce more than one mycotoxin (particularly

Fusarium spp.), food commodities can be contaminated by several fungi, and animal and human diets usually consist of multiple commodities.

116. An external EFSA report by Battilani *et al.*, (2020) was published; the group carried out an extensive literature review whereby a platform was built named MYCHIF. The database is comprised of four topics including: the ecological background of mycotoxins and their interactions with host plants, the available analytical methods to detect the co-occurrence of mycotoxins, the toxicological and biomarkers data relevant to humans and animals and modelling approaches in order to perform risk modelling.

117. Using the gathered information, a case study was carried out for two mycotoxin mixtures (1: DON, FBs and ZEN and 2: T-2/HT-2 toxin, DON and NIV). Biomarker data was utilised as the basis for the exposure assessment which was carried out probabilistically either with a component-based and provisional daily intake approaches.

118. For the CBA an MOE of <100 for all age groups (adolescent, adult and elderly) was calculated, where as a hazard index exposure index could not be calculated for the provisional daily intake approach. A hierarchy map (based on EU member states) for adults was compiled and provided a visual representation of higher risked exposure groups to T2/HT-2 toxin, DON and NIV.

119. With reference to the co-occurrence and occurrence data collected for the United Kingdom (UK); T2/HT-2 toxin, DON and NIV adult exposure levels based on the mean co-occurrence data and mean consumption value were >0.8 ,0.6-1.2 and 0.24-0.3 µg/kg bw/day, respectively.

120. To put into context the above exposure values against the HBGV for each single mycotoxin (see paragraph 32 for values), the estimated exposure levels for UK adults of T2/HT-2 toxin is above the HBGV, whilst exposure levels for DON and NIV are below their respective HBGVs.

121. In terms of data gaps, there is still a limited knowledge on the presence and co-occurrence of multiple mycotoxins, both for primary mycotoxins and their modified forms, in food and feed since current analytical methods have limitations. Furthermore, there is a limited number of toxicity data and there is a lack of consensus on methodologies and guidelines for generating *in vitro* and *in vivo* TK and TD. Model definitions are also required for the utilisation of biomarkers for exposure assessments.

122. Other opinions by authoritative groups on some mycotoxin mixtures were also summarised. The EFSA CONTAM Panel concluded that the available data for interactions between 4,15-DAS and other mycotoxins (T-2 and HT-2 toxins, AFs, OTA and FBs) is weak and inconclusive (EFSA, 2018). In contrast, the EFSA CONTAM Panel concluded that the combined effect of CIT and OTA is at most additive (EFSA, 2012). The JECFA Committee concluded that even though there are additive or synergistic effects observed from FB1 and AFB1 co-exposure in laboratory animals in inducing the

development of preneoplastic lesions or hepatocellular carcinoma (Torres *et al*., 2015; Carlson *et al*., 2001; Gelderbloom *et al*., 2002), there was currently no data available on such effects in humans. The combined toxicity of FBs and DON were suggestive of being additive or more than additive, however, the observed effect is dependent on the endpoints measured (JECFA, 2018).

123. The main analytical methods to measure mycotoxins in food commodities are: ELISA, GC and LC-MS, whilst LC–MS/MS is the main analytical method for detecting and measuring co-occurrence of very low concentrations of mycotoxins, however, these advanced multi-mycotoxin techniques are not yet commonly applied in routine screening analyses due to their associated high cost.

124. In terms of toxicokinetic data, only one human study was identified to have investigated and analysed the combined exposure to DON and ZEN (Warth *et al*., 2013). This study, however, had its limitations which was mainly due to the number of volunteers (n=1 male) and in effect, does not cover inter-individual variations. It is hypothesised that the toxicokinetics of mycotoxin mixtures may need to be addressed on a case-by-case approach; however, it is recognised that the mycotoxin dose, exposure pathway, interspecies and intraspecies differences are identified as the most influential parameters that may affect observations.

125. The toxic effects of some binary mycotoxins were discussed in this paper (*e.g*. AFB1 and FB1, OTA and DON *etc*., see *Tables 3 - 5*). The availability of *in vivo* data directly relevant for humans is scarce with most studies only covering a limited number of mycotoxin combinations and more generally focused on animal models of agricultural importance *i.e*. pigs and chickens.

126. The toxicity of combinations cannot be predicted based on the toxicity of individual mycotoxins. Furthermore, the comparison of studies may prove to be challenging since there is a large amount of variability between each methodology carried out due to the lack of harmonisation on combinative testing strategies for each toxicological endpoint.

127. In terms of exposure assessments, the use of biomarkers data was explored in the MYCHIF report by Battilani *et al*., (2020) (see paragraphs 27-31 and 81-86). It was concluded that the use of biomarkers may be premature due since there is a lack of: knowledge on the human bioavailability of the toxin combination, and the excretion rate. There are also several limitations associated with multi-biomarker monitoring. These include:

i). Biological fluids contain extremely low analyte concentrations following dietary exposure, as such sample preparation is crucial to obtain acceptable limit of detection;
ii). There is a great chemical diversity of analytes and this makes clean-up methodologies challenging (*e.g*. polar compounds like glucuronides);

iii). Careful optimisation needs to be carried out to overcome matrix effects and interfering matrix peaks, eluents, the chromatographic gradient, and the dilution factor;

iv). The co-elution of matrix components is said to have a negative influence on the accuracy of quantitative methods through ion suppression or enhancement in the ion source and;

v). In general, there is a lack of authentic reference standards and certified reference materials and a consensus of a validated biomarker to be used in context with multi-mycotoxin analyses.

128. A suggested major research gap was the investigation of potential concurrent exposure of mycotoxins with other environmental chemicals that may exhibit some interactive activity and/or exert a biological function converging in the same molecular pathways.

129. A stepwise approach for the exposure assessment was detailed (see paragraphs 72-75) in this paper, these were based on the used the on use of deterministic and if necessary, probabilistic approaches. Three data sources were also identified to use in the exposure assessment, these were the FSA TDS Mycotoxin Study (Stratton *et al*., 2017), the MYCHIF platform (Battilani *et al*., 2020), and exposure data derived from literature.

130. Based on the limitations presented throughout this document and the absence of an exposure assessment, a full risk assessment on the potential adverse effects of combined dietary exposure to mycotoxins could not be performed by the COT.

COT conclusions and recommended research

131. As noted above, a risk assessment could not be carried out on the potential risks to combined exposure of mycotoxins at the time of the COT review for several reasons. There is a lack of harmonisation of approaches/methodologies and data analysis/modelling for toxicological investigations. Additionally, the underlying mechanisms of interactions between each mycotoxin combination is yet to be fully elucidated and understood. Further considerations for risk assessment include the potential toxic effects of mycotoxin mixtures on the gut microbiota and the endocrine system. Co-exposures from breastmilk and weaning foods must also be considered for infants and young children.

132. Furthermore, the availability of food consumption data is scarce, and the development of multi-analyte methods is still not yet fully applied as standard. Lastly, the management of left-censored data, the use of probabilistic models and a multi-biomarker approach should be consistent and have a well-defined approach.

133. The COT noted that there was a lack of UK data, particularly in biomonitoring; however, there were a number of studies ongoing and new information will be available in the future. The Public Health England

Secretariat informed COT Members that the UK will not be collecting new data for mycotoxins under the HBM4EU initiative. However, in the future, more data could be obtained through Health Protection Research Units. Such research was considered to be a priority by the COT.

134. Members recommended as a pragmatic first step that a review should be carried out of the compounds which appeared to show a common effect on protein synthesis, assuming dose additivity, and that they frequently co-occur in food commodities – an exposure estimate can be performed and can be compared to HBGVs (to calculate the MOE) or utilise HI to determine whether there is any potential concern from co-exposure to mycotoxins.

135. Research is needed on mycotoxins affecting ribosomal protein synthesis to determine whether they do exhibit dose additivity in their effects, to help develop a reliable basis for their cumulative risk assessment.

COT
January 2021

Abbreviations

15-AcDON	15-acetyldeoxynivalenol
3-AcDON	3-acetyldeoxynivalenol
4,15-DAS	4,15-diacetoxyscirpenol
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFM1	Aflatoxin M1
AFs	Aflatoxins
ARfD	Acute reference dose
CBA	Component-based approach
CIT	Citrinin
CONTAM	Panel on Contaminants in the Food Chain
COPHES	Consortium to Perform Human Biomonitoring on a European Scale
COT	Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
DNA	Deoxyribonucleic acid
DOM-1	De-epoxy deoxynivalenol
DON	Deoxynivalenol
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ENN	Enniatins
FB1	Fumonisin B1
FBs	Fumonisins
Fus-X	Fusarenon-X
GC-MS	Gas chromatography-mass spectroscopy
HBGV	Health based guidance value
HBM	Human biomonitoring
HBM4EU	European Union Human Biomonitoring
HELIX	Human Early-Life Exposome
HPLC	High-performance liquid chromatography
JECFA	Joint Food and Agriculture Organization and World Health Organisation Expert Committee on Food Additives
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectroscopy
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MOA	Mode of action
MOE	Margin of exposure
MYCHIF	Mycotoxin Mixtures in Food and Feed: Holistic, Innovative, Flexible Risk Assessment Modelling Approach
MYCOMIX	Mycotoxin mixtures
MYTOX	Mycotoxin and Toxigenic Moulds
NIV	Nivalenol
OTA	Ochratoxin A
PAT	Patulin
SCF	Scientific Committee on Food
TD	Toxicodynamic
TDI	Tolerable daily intake
TDS	Total diet study

TK	Toxicokinetic
UK	United Kingdom
ZEN	Zearalenone

References

Abdel-Wahhab, M. A., El-Denshary, E. S., El-Nekeety, A. A., Abdel-Wahhab, K. G., Hamzawy, M. A., Elyamany, M. F., Hassan, N. S., Mannaa, F. A., Shaiea, M. N. and Gado, R. A. (2015) Efficacy of organo-modified nano montmorillonite to protect against the cumulative health risk of aflatoxin B1 and ochratoxin A in rats. Soft Nanoscience Letters 5, 21.

Agriopoulou, S., Stamatelopoulou, E. and Varzakas, T. (2020) Advances in Analysis and Detection of Major Mycotoxins in Foods. Foods 9, 518.

Alassane-Kpembi, I., Kolf-Clauw, M., Gauthier, T., Abrami, R., Abiola, F.A., Oswald, I.P. and Puel, O. (2013) New insights into mycotoxin mixtures: The toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic. Toxicology and Applied Pharmacology 272, pp. 191-198.

Alassane-Kpembi, I., Schatzmayr, G., Taranu, I., Marin, D., Puel, O. and Oswald, I. P. (2017) Mycotoxins co-contamination: methodological aspects and biological relevance of combined toxicity studies. Critical Reviews in Food Science and Nutrition 57, pp. 3489 –3507.

Alvito, P., Assunção, R., Borges, T., Leal, S., Loureiro, S., Louro, H., Martins, C., Nunes, B., Silva, M., Vasco, E., Tavares, A. and Calhau, M. (2015) Risk assessment of multiple mycotoxins in infant food consumed by Portuguese children – the contribute of the MYCOMIX project. Toxicology Letters 238: S117.

Assunçao, R., Martins, R., Vasco, E., Jager, A., Oliveira, C., Cunha, S. C., Fernandes, J. O., Nunes, B., Loureiro, S. and Alvito. P. (2018) Portuguese children dietary exposure to multiple mycotoxins – An overview of risk assessment under MYCOMIX project. Food and Chemical Toxicology 118, pp. 399-408.

Baines, D., Sumarah, M., Kuldau, G., Juba, J., Mazza, A. and Masson, L. (2013) Aflatoxin, Fumonisin and Shiga Toxin-Producing Escherichia coli infections in calves and the effectiveness of Celmanax®/Dairyman's Choice™ applications to eliminate morbidity and mortality losses. Toxins 5, pp. 1872-1895.

Battilani, P., Palumbo, R., Giorni, P., Dall'Asta, C., Dellafiora, L., Gkrillas, A., Toscano, P., Crisci, A., Brera, C., De Santis, B., Cammarano, R. R., Della Seta, M., Campbell, K., Elliot, C., Venancio, A., Lima, N., Gonçalves, A., Terciolo, C. and Oswald, I. P. (2020) Mycotoxin mixtures in food and feed: holistic, innovative, flexible risk assessment modelling approach: MYCHIF. EFSA External Scientific Report. https://doi.org/10.2903/sp.efsa.2020.EN-1757.

Bensassi, F., Gallerne, C., Hajlaoui, M. R., Lemaire, C. and Bacha, H. (2014) In vitro investigation of toxicological interactions between the fusariotoxins deoxynivalenol and zearalenone. Toxicon 84, pp. 1−6.

Bernhoft, A., Keblys, M., Morrison, E., Larsen, H.J.S. and Flaoyen, A. (2004) Combined effects of selected Penicillium mycotoxins on in vitro proliferation of porcine lymphocytes. Mycopathologia 158, pp. 441-450.

Bessaire, T., Mujahid, C., Mottier, P. and Desmarchelier, A. (2019) Multiple Mycotoxins Determination in Food by LC-MS/MS: An International Collaborative Study. Toxins 11, 658.

BIOMIN. (2020a) BIOMIN Mycotoxin Survey Q3 January to September 2020 Results. Available at: https://www.biomin.net/science-hub/biomin-mycotoxin-survey-q3-2020-results//. Accessed: 05/01/2021.

BIOMIN. (2020b) BIOMIN World Mycotoxin Survey the Global Threat January – June 2020. Available at: https://www.biomin.net/science-hub/biomin-mycotoxin-survey-q3-2020-results/. Accessed: 05/01/2021.

Bouslimi, A., Bouaziz, C., Ayed-Boussema, I., Hassen, W. and Bacha, H. (2008) Individual and combined effects of ochratoxin A and citrinin on viability and DNA fragmentation in cultured Vero cells and on chromosome aberrations in mice bone marrow cells. Toxicology 251, pp. 1-7.

Bracarense, A. P. F., Lucioli, J., Grenier, B., Pacheco, G. D., Moll, W. D., Schatzmayr, G. and Oswald, I. P. (2012) Chronic ingestion of deoxynivalenol and fumonisin, alone or in interaction, induces morphological and immunological changes in the intestine of piglets. British Journal of Nutrition 107, pp. 1776−1786.

Braicu, C., Berindan-Neagoe, I., Chedea, V.S., Balacescu, L., Brie, I., Soritau, O., Socaciu, C. and Irimie, A. (2010) Individual and combined cytotoxic effects of the major four aflatoxins in different in vivo stabilised systems. Journal of Food Biochemistry 34, pp. 1079-1090.

Carlson, D. B., Williams, D. E., Spitsbergen, J. M., Ross, P. F., Bacon, C. W. and Meredith, F. I. (2001). Fumonisin B1 promotes aflatoxin B1 and N-methyl-N'-nitro-nitrosoguanidine initiated liver tumors in rainbow trout. Toxicology and Applied Pharmacology 172, pp. 29–36.

Corcuera, L. A., Arbillaga, L., Vettorazzi, A., Azqueta, A. and de Cerain, A. L. (2011) Ochratoxin A reduces aflatoxin B1 induced DNA damage detected by the comet assay in HepG2 cells. Food and Chemical Toxicology 49, pp. 2883−2889.

Creppy, E. E., Chiarappa, P., Baudrimont, I., Borracci, P., Moukha, S. and Carratù, M. R. (2004) Synergistic effects of fumonisin B1 and ochratoxin A: Are in vitro cytotoxicity data predictive of in vivo acute toxicity? Toxicology 20, pp. 115−123.

De Ruyck, K., Boevre, M., Huybrechts, I. and De Saeger, S. (2015) Dietary mycotoxins, co-exposure, and carcinogenesis in humans: short review. Mutation Research-Reviews in Mutation Research, 766, pp. 32-41.

De Santis, B., Debegnach, F., Gregori, E., Russo, S., Marchegiani, F., Moracci, G. and Brera, C. (2017) Development of a LC-MS/MS method for the multi-mycotoxin determination in composite cereal-based samples. Toxins 9, 169.

De Saeger, S. (2021) Personal communication.

Demaegt, H., Daminet, B., Evrard, A., Scippo, M-L., Muller, M., Pussemier, L., Callebaut, and Vandermeiren, K. (2016) Endocrine activity of mycotoxins and mycotoxin mixtures. Food and Chemical Toxicology 96, pp. 107-116.

Domijan, A. M., Želježić, D., Kopjar, N. and Peraica, M. (2006) Standard and Fpg-modified comet assay in kidney cells of ochratoxin A and fumonisin B1 treated rats. Toxicology 222, pp. 53−59.

EFSA. (2010) Scientific Report on the management of left-censored data in dietary exposure assessment of chemical substances. Available at: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2010.1557.

Accessed: 04/01/2021.EFSA. (2012a) Scientific Opinion on the risks for public and animal health related to the presence of citrinin in food and feed. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/2605. Accessed: 05/10/2020.

EFSA. (2012b) Statement on the applicability of the Margin of Exposure approach for the safety assessment of impurities which are both genotoxic and carcinogenic in substances added to food/feed. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/2578. Accessed: 09/10/2020.

EFSA. (2013) Scientific Opinion on risks for animal and public health related to the presence of nivalenol in food and feed. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/3262. Accessed: 09/10/2020.

EFSA. (2017a) Appropriateness to set a group health-based guidance value for T2 and HT2 toxin and its modified forms. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4655. Accessed: 08/10/2020.

EFSA. (2017b) Scientific opinion on the risk to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4718. Accessed: 05/10/2020.

EFSA. (2018) Risk to human and animal health related to the presence of 4,15-diacetoxyscirpenol in food and feed. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/5367. Accessed: 05/10/2020.

EFSA. (2019) Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/5634. Accessed: 11/01/2021.

Ficheux, A., Sibiril, Y. and Parent-Massin, D. (2012) Co-exposure of Fusarium mycotoxins: *in vitro* myelotoxicity assessment on human hematopoietic progenitors. Toxicon 60, pp. 1171−1179.

Flores-Flores, M. E. and González-Peñas, E. (2017) An LC-MS/MS method for multi-mycotoxin quantification in cow milk. Food Chemistry 218, pp. 378-385.

Freire, L. and Sant'Ana, A. S. (2018) Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. Food and Chemical Toxicology 111, pp. 189-205.

Friedman, L., Gaines, D. W., Chi, R. K., Smith, M. C., Braunberg, R. C. and Thorpe, C. W. (1997) Interaction of aflatoxins as measured by their biochemical action on rat liver slices and hepatocytes. Toxic Substance Mechanisms 16, pp. 15-41.

Gambacorta, L., Magistà, D., Perrone, G., Murgolo, S., Logrieco, A. F., Solfrizzo, M. (2018) Co-occurrence of toxigenic moulds, aflatoxins, ochratoxin A, *Fusarium* and *Alternaria* mycotoxins in fresh sweet peppers (Capsicum annuum) and their processed products. World Mycotoxin Journal 11, pp. 159-173.

Gelderblom, W., Marasas, W., Lebepe-Mazur, S., Swanevelder, S., Vessey, C. and Hall, P. de L. (2002) Interaction of fumonisin B1 and aflatoxin B1 in a short-term carcinogenesis model in rat liver. Toxicology 171, pp. 161−173.

Golli-Bennour, E. E., Kouidhi, B., Bouslimi, A., Abid-Essefi, S., Hassen, W. and Bacha, H. (2010) Cytotoxicity and genotoxicity induced by aflatoxin B1, ochratoxin A, and their combination in cultured Vero cells. Journal of Biochemical and Molecular Toxicology 24, pp. 42−50.

Grenier, B. and Oswald, I. P. (2011) Mycotoxin co-contamination of food and feed: Meta-analysis of publications describing toxicological interactions. World Mycotoxin Journal 4, pp. 285-313.

JECFA. (2011) Evaluation of certain food additives and contaminants. Seventy-fourth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series 966. (pp.76). Available at: https://apps.who.int/iris/bitstream/handle/10665/44788/WHO_TRS_966_eng.pdf;jsessionid=28FF91FD12C2F291487CE9DB9371E60E?sequence=1. Accessed: 05/10/2020.

JECFA. (2018) Safety evaluation of certain contaminants in food. WHO Food Additive Series: 74. Prepared for the eighty-third meeting of the Joint FAO/WHO Expert Committee on Foods and Additives (JECFA). (pp. 463-464). Available at: http://www.inchem.org/documents/jecfa/jecmono/v74je01.pdf. Accessed: 09/10/2020.

Kouadio, J. H., Lattanzio, V. M. T., Ouattara, D., Kouakou, B., Visconti, A. (2014) Assessment of mycotoxin exposure in Côte d'Ivoire (Ivory Coast) through multi-biomarker analysis and possible correlation with food consumption patterns. International Journal of Toxicology 21, pp. 248–57.

Krska, R., Schubert-Ullrich, P., Molinelli, A., Sulyok, M., MacDonald, S. and Crews, C. (2007) Mycotoxin analysis: An update. Food Additives & Contaminants: Part A 25, pp. 152-163.

Lee, H, J & Ryu, D. (2017) Worldwide occurrence of mycotoxins in cereals and cereal-derived food products: Public health perspectives of their co-occurrence. Journal of Agricultural and Food Chemistry 65, pp. 7034-7051.

Lei, M., Zhang, N. and Qi, D. (2013) In vitro investigation of individual and combined cytotoxic effects of aflatoxin B1 and other selected mycotoxins on the cell line porcine kidney 15. Experimental and Toxicologic Pathology 2013, 65, pp. 1149−1157.

Li, Y., Zhang, B., He, X., Cheng, W. H., Xu, W., Luo, Y., Liang, R., Luo, H. and Huang, K. (2014) Analysis of individual and combined effects of ochratoxin A and zearalenone on HepG2 and KK-1 cells with mathematical models. Toxins 6, pp. 1177−1192.

Liew, W-P-P. and Mohd-Redzwan, S. (2018). Mycotoxin: Its impact on gut health and microbiota. Frontiers in Cellular and Infection Microbiology 8, 60.

MacDonald, S. (2020) Personal communication.

Magoha, H., Kimanya, M., De Meulenaer, B., Roberfroid, D., Lachat, C. and Kolsteren, P. (2016) Risk of dietary exposure to aflatoxins and fumonisins in infants less than 6 months of age in Rombo, northern Tanzania. Journal of Maternal and Child Nutrition 12, pp. 516–27.

Malachová, A., Sulyok, M., Beltrán, E., Berthiller, F., Krska, R. (2014) Optimization and validation of a quantitative liquid chromatography-tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices. Journal of Chromatography A 1362, pp. 145-156.

Marín, S., Cano-Sancho, G., Sanchis, V. and Ramos, A.J. (2018) The role of mycotoxins in the human exposome: Application of mycotoxin biomarkers in exposome-health studies. Food & Chemical Toxicology 121, pp.504–518.

Mary, V. S., Theumer, M. G., Arias, S. L. and Rubinstein, H. R. (2012) Reactive oxygen species sources and biomolecular oxidative damage induced by aflatoxin B1 and fumonisin B1 in rat spleen mononuclear cells. Toxicology 302, pp. 299−307.

McKean, C., Tang, L., Tang, M., Billam, M., Wang, Z., Theodorakis, C.W., Kendall, R.J. and Wang, J.S. (2006a) Comparative acute and combinative toxicity of aflatoxin B1 and fumonisin B1 in animals and human cells. Food and Chemical Toxicology 44, 868-876.

McKean, C., Tang, L., Billam, M., Tang, M., Theodorakis, C.W., Kendall, R.J. and Wang, J.S. (2006b) Comparative acute and combinative toxicity of aflatoxin B1 and T-2 toxin in animals and immortalized human cell lines. Journal of Applied Toxicology 26, pp. 139-147.

Mwanza, M., Kametler, L., Bonai, A., Rajli, V., Kovacs, M. and Dutton, M. F. (2009) The cytotoxic effect of fumonisin B1 and ochratoxin A on human and pig lymphocytes using the methyl thiazol tetrazolium (MTT) assay. Mycotoxin Research 25, pp. 233−238.

Orsi, R., Oliveira, C., Dilkin, P., Xavier, J., Direito, G. and Correa, B. (2007) Effects of oral administration of aflatoxin B1 and fumonisin B1 in rabbits (Oryctolagus cuniculus). Chemico-Biological Interactions 170, pp. 201−208.

Palumbo, R., Crisci, A., Venâncio, A., Abrahantes, J. C., Dorne, J., Battilani, P. and Toscano, P. (2020) Occurrence and co-occurrence of mycotoxins in cereal-based feed and food. Microorganisms 8, 74.

Qian, G., Lili, L., Shuhan, L., Xue, K. S., Mitchell, N. J., Su, J., Gelderblom, W. C., Riley, R. T., Phillips, T. D. and Wang, J. (2016). Sequential dietary exposure to aflatoxin B1 and FB1 in F344 rats increases preneoplastic changes indicative of a synergistic interaction. Food and Chemical Toxicology 95, pp. 188–195.

Riley, R. T., Hambridge, T., Alexander, J., Boon, P. E., Doerge, D. R., Edwards, S., Miller, D., Shephard, G. S. and Zhang, Y. (2018) Co-exposure of fumonisins with aflatoxins. Available at: http://www.inchem.org/documents/jecfa/jecmono/v74je01.pdf (pp. 879-957). Accessed: 05/10/2020.

SCF. (2002) Opinion of the Scientific Committee on Food on *Fusarium* toxins. Part 6: Group evaluation of T-2 toxin, HT-2 toxin, nivalenol and Deoxynivalenol. SCF/CS/CNTM/MYC/27 Final. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_catalogue_fusarium_out123_en.pdf. Accessed: 10/10/2020.

Sedmíková, M., Reisnerova, H., Dufkova, Z., Barta, I. and Jilek, F. (2001) Potential hazard of simultaneous occurrence of aflatoxin B-1 and ochratoxin A. Vet Med-Czech 46, pp. 169-174.

Šegvić Klarić, M., Pepeljnjak, S., Domijan, A. M. and Petrik, J. (2007) Lipid peroxidation and glutathione levels in porcine kidney PK15 cells after individual and combined treatment with fumonisin B1, beauvericin and ochratoxin A. Basic & Clinical Pharmacology & Toxicology 100, pp. 157−164.

Šegvić Klarić, M., Zeljezic, D., Rumora, L., Peraica, M., Pepeljnjak, S. and Domijan, A.M. (2012) A potential role of calcium in apoptosis and aberrant chromatin forms in porcine kidney PK15 cells induced by individual and combined ochratoxin A and citrinin. Archives of Toxicology 86, pp. 97-107.

Serrano, A. B., Font, G., Ruiz, M. J. and Ferrer, R. E. (2012) Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area. Food Chemistry 135, pp. 423-429.

Shi, H., Schwab, W. and Yu. P. (2019) Natural occurrence and co-contamination of twelve mycotoxins in industry-submitted cool-season cereal grains grown under a low heat unit climate condition. Toxins 11, 160.

Shirima, C. P., Kimanya, M. E., Routledge, M. N., Srey, C., Kinabo, J. L., Humpf, H. U, Wild, C. P., Tu, Y-K. and Gong, Y. Y. (2015) A prospective study of growth and biomarkers of exposure to aflatoxin and fumonisin during early childhood in Tanzania. Environ Health Perspectives 23, pp. 173–178.

Singh, J. and Mehta, A. (2020) Rapid and sensitive detection of mycotoxins by advanced and emerging analytical methods: A review. Food Science & Nutrition 8, pp. 2183-2204.

Šmerák, P., Barta, I., Polivkova, Z., Bartova, J. and Sedmíková, M. (2001) Mutagenic effects of selected trichothecene mycotoxins and their combinations with aflatoxin B1. Czech Journal of Food Sciences 19, pp. 90-96.

Smith, M-C., Madec, S., Coton, E. and Hymery, N. (2016) Natural co-occurrence of mycotoxins in foods and feeds and their *in vitro* combined toxicological effects. Toxins 8, 94.

Speijers, G. J. A. and Speijers, M. H. M. (2004) Combined toxic effects of mycotoxins. Toxicology Letters 153, pp. 91-98.

Stratton, J., Anderson, S., Leon, I., Hepworth, P., Chapman, S., Christy, J., Jardine, S., Philips, D., Setter, R., Clough, J. and MacDonald, S. (2017) Final Report Diet Study (TDS) – Mycotoxin analysis. FS102081. Available at: https://www.food.gov.uk/sites/default/files/media/document/final-report-diet-study-tds-survey-mycotoxins.pdf. Accessed: 09/10/2020.

Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Marin, D., Taranu, I., Tabuc, C., Nicolau, A., Aprodu, I., Puel, O. and Oswald, I. P. (2012) Current situation of mycotoxin contamination and co-occurrence in animal feed – Focus on Europe. Toxins 4, pp. 788-809.

Sun, L. H., Lei, M. Y., Zhang, N. Y., Gao, X., Li, C., Krumm, C. S. and Qi, D. S. (2015) Individual and combined cytotoxic effects of aflatoxin B1, zearalenone, deoxynivalenol and fumonisin B1 on BRL 3A rat liver cells. Toxicon 95, pp. 6−12.

Theumer, M. G., López, A. G., Aoki, M. P., Cánepa, M. C. and Rubinstein, H. R. (2008) Subchronic mycotoxicoses in rats. Histopathological changes and modulation of the sphinganine to sphingosine (Sa/So) ratio imbalance induced by Fusarium verticillioides culture material, due to the coexistence of aflatoxin B1 in the diet. Food and Chemical Toxicology 46, pp. 967-977.

Torres, O., Matute, J., Gelineau-van Waes, J., Maddox, J. R., Gregory, S. G. and Ashley-Koch, A. E. (2015) Human health implications from co-exposure to aflatoxins and fumonisins in maize-based foods in Latin America: Guatemala as a case study. World Mycotoxin Journal 8, pp. 143–59.

Wang, H. W., Wang, J. Q., Zheng, B. Q., Li, S. L., Zhang, Y. D., Li, F. D. and Zheng, N. (2014) Cytotoxicity induced by ochratoxin A, zearalenone, and α-zearalenol: Effects of individual and combined treatment. Food and Chemical Toxicology 71, pp. 217-224.

Wangikar, P. B., Dwivedi, P. and Sinha, N. (2004) Effects of rats of simultaneous prenatal exposure to ochratoxin A and aflatoxin B1. I. Maternal toxicity and fetal malformations. Birth Defects Research (Part B) 71, pp. 343-351.

Wangikar, P. B., Dwivedi, P. and Sinha, N. (2004) Effects of rats of simultaneous prenatal exposure to ochratoxin A and aflatoxin B1. II. Maternal toxicity

Warth, B., Sulyok, M., Berthiller, F., Schuhmacher R. and Krska, R. (2013) New insights into the human metabolism of the Fusarium mycotoxins deoxynivalenol and zearalenone. Toxicology Letters 220, pp. 88-94

WHO. (2018) Mycotoxins. Available at: https://www.who.int/news-room/fact-sheets/detail/mycotoxins. Accessed: 02/10/2020.

Yang, Y., Yun, S., Tan, Y., Liu, N., Wu, A. (2017) Individual and combined cytotoxic effects of co-occurring deoxynivalenol family mycotoxins on human gastric epithelial cells. Toxins 9, 96.