TOX/2020/34

Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT)

Potential risks from aggregated dietary exposure to mycotoxins

Background

1. The potential risks from co-exposure to mycotoxins was identified as a topic that the COT should consider during horizon scanning.

2. Climate change is thought to have a significant impact on the stages and rates of the development of toxicogenic fungi and has the ability to modify host-resistance and host-pathogen interactions. In turn, this will influence the conditions for mycotoxin production that varies for each individual pathogen.

3. In light of this, new combinations of factors (mycotoxins/host plants and geographical location) will have to be considered when assessing the potential risks of co-exposure to mycotoxins. This scoping paper will provide an overview of the methods of estimating the combined exposures to mycotoxins and subsequently their human health related effects.

4. The COT has previously reviewed mycotoxins in the diets of infants aged 0-12 months and children aged 1-5 years, with the initial discussion paper presented in 2017 (TOX/2017/30)[1]. Since then, the overarching statement has been published in April 2020[2] which concluded the infant and children 0-5 review. A lay summary was also made available[3].

5. The European Food Safety Authority (EFSA) have recently published an external scientific report on mycotoxin mixtures in food and feed in January 2020 (Battilani *et al*., 2020)[4], which will form the basis of this review. Additionally, there are several study groups that have and are carrying out ongoing research in this field. Furthermore, a literature search was carried out from 2000 to current, in order to gain insight and identify the risks or trends that might arise from the aggregated dietary exposure to mycotoxins.

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[1] TOX/2017/30 available on the COT website.
[2] 0-5 overarching statement available on the COT website.
[3] 0-5 overarching statement (lay summary) available on the COT website.
[4] EFSA external scientific report on mycotoxin mixtures in food and feed available on the EFSA website.

Introduction

6. Mycotoxins are toxic secondary metabolites produced by fungi and can cause adverse health effects in both humans and animals. Cereals are often the most severely affected crops; however, some nuts, fruits and spices can also be affected. Mould growth can occur either pre- or post-harvest, during storage, on/in the food itself under warm, damp, and humid conditions. Mycotoxins are stable low-molecular weight chemicals and are not often affected by food processing.

7. Mycotoxins of greatest concern to human health and livestock are produced by several fungal 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).

8. 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 (Smith, 2016).

9. 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 weed and pest. 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 (Battilani *et al*., 2020).

10. Mycotoxin colonisation also depends on temperature, relative humidity, rainfall and water activity. 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. Exposure to dietary mycotoxins can lead to several adverse effects in humans, which include carcinogenic, teratogenic, hepatotoxic, nephrotoxic, and cytotoxic effects, as well as immunological and haematological disturbances.

12. The main human and veterinary health burdens of mycotoxin exposure are related to chronic exposure (cancer, kidney damage, immune suppression), whilst the manifestations of acute effects observed in animals and humans include Turkey X-syndrome and human ergotism, respectively (De Ruyck *et al*., 2015).

13. Some mycotoxins are able to resist decomposition by digestion in ruminant livestock, as such, these compounds persist in end products like meat and dairy. Mycotoxins are not inactivated by temperature treatments, such as cooking and

freezing, making it practically impossible to eliminate them in already contaminated foods.

14. As mentioned, the COT has previously reviewed the risks of single exposures of mycotoxins in infants and children (TOX/2017/30[5] and the 0-5 years overarching statement[6]). This current paper will address the potential risks of aggregate dietary exposure from mycotoxins for all age groups (where data is available).

Search strategy

15. The following search strategies were combined to identify literature relevant to the co-exposure and toxicity of mycotoxins to humans. PubMed, Science Direct and Google Scholar databases were searched using single words or combinations of terms as described in Annex A.

Co-occurrence of mycotoxins

16. 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.

17. Palumbo *et al*., (2020) carried out a recent review on the occurrence and co-occurrence of mycotoxins (*i.e.* parent and modified forms) in European core cereals. Mycotoxins were mainly reported in wheat and maize. In decreasing order, the highest detected levels were FBs, DON, AFB_1, AFB_2 and ZEN. Exceedances of DON with respect to its regulatory limits in wheat, barely, maize and oat were observed (750 µg/kg). FBs widely reported in maize foods and feed also exceeded its regulatory limits of 1,000 and 4,000 µg/kg, respectively. Co-occurrence (*i.e.* cereals were co-contaminated with at least two mycotoxins) was reported in 54.9% of total records (n=206). The co-occurrence of DON was frequently observed with FBs in maize and ZEN in wheat, whilst the combination of DON and NIV were frequently reported in barley. DON and T-2/HT-2 toxins were also frequently reported in oat. In terms of the detection of co-occurrence with modified forms, only a limited number of quantified data were available.

18. It was concluded by Palumbo *et al*., (2020) that wheat and maize may contribute significantly to mycotoxin co-exposure in human and animal species

[5] TOX/2017/30 available on the COT website.
[6] 0-5 years overarching statement available on the COT website.

compared to other crops. Additionally, there is still limited knowledge on the presence and co-occurrence of multiple mycotoxins, both for native mycotoxins and their modified forms, in food and feed (NB. This review only considered European core cereals). The authors presented the following recommendations:

i). That continuous monitoring of major mycotoxins in different agricultural commodities and a harmonised methodology for generating accurate co-occurrence data is set-up;
ii). The management of left-censored data for mycotoxins should be further harmonised as it presents a major source of uncertainty when assessing the exposure to multiple chemical substances and;
iii). A more accurate reporting of geographical information of samples could further optimise efforts to better understand and map the mycotoxin problem in the European Union (EU).

19. Streit *et al*., (2012) focused their efforts on reviewing the data published since 2004 concerning the contamination of animal feed with single or combinations of mycotoxins (in Europe) and highlighted the occurrence of these co-contaminations. It was identified that only a limited number of mycotoxins are subject to regular testing and legal regulations/guidance, namely AFs, DON, ZEN, FBs and OTA.

20. Reviewed literature showed that mycotoxins were present ubiquitously in feed material throughout Europe and that maximum contamination levels exceeding the permitted regulation/guidance levels are likely to occur. Low level contamination of *Fusarium* toxins (*e.g.* DON) was common in more than 50% of the samples, and co-contamination is frequently observed with some multi-mycotoxin studies reporting 75-100% of samples to contain more than one mycotoxin.

21. In this review, United Kingdom (UK) specific data by Scudamore *et al*., (1998) discussed maize products for animal feed which had been screened for 22 mycotoxins. It was found that all samples (n=67) were co-contaminated with up to 12 different *Fusarium* mycotoxins. FBs and DON occurred together in 75% of the samples and 15-acetlydeoxynivalenol (15-acDON), moniliformin, and ZEN were frequent co-contaminants. None of the samples contained detectable amounts of AFBs, whilst the previously study the same group observed contamination of AF in maize at 14%, with FBs being the most common at 28% (n=330 samples) (Scudamore *et al*., 1997).

22. The authors added that it was difficult to infer trends or recent developments regarding mycotoxin contamination in European feed from the reviewed 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, which included

enzyme-linked immunosorbent assay (ELISA), thin layer chromatography, gas chromatography (GC), and high performance liquid chromatography (HPLC). Furthermore, sampling methodologies were not often described in detail, and it was considered by the authors to be the largest source of error and variability in mycotoxin analysis.

23. The authors recommended that continuous monitoring was needed in order to avoid negative impacts on animal health and performance due to elevated contamination levels, which may occur, for example, after growing seasons characterised by weather conditions particularly favourable to fungal infection and growth. The development of predictive models of mycotoxin occurrence based on regional weather data would also prove to be a valuable tool to estimate the risk of contamination after a given growing season. Increasing farmer's awareness of the issue of mycotoxins would also prove to be beneficial. For example, the application of mycotoxin reduction strategies such as the addition of mycotoxin deactivating products based on different strategies (*i.e.* adsorption, biotransformation, biodegradation, bio-protection) should be considered. Lastly, the development and application of multi-mycotoxin liquid chromatography with tandem mass spectroscopy (LC-MS/MS) methods should be encouraged in order to get a more accurate picture of the extent of multi-mycotoxin contamination.

Methods for sampling and measuring mixtures of mycotoxins in food matrices

24. The main analytical methods to measure mycotoxins are ELISA, GC-MS and LC-MS. Currently, liquid chromatography based methods are the most frequently used (Serrano *et al*., 2012; Malachová *et al*., 2018), with several mass spectrometric detectors such as fluorescence detector, 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.

25. 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)

26. The main analytical method used for detecting and measuring co-occurrence of very low concentrations of mycotoxins is LC–MS/MS (Serrano *et al*., 2012; Shi *et al*., 2019; Malachová *et al*., 2018; Palumbo *et al*., 2020; Gambacorta *et al*., 2018; Battliani *et al*., 2020). The compounds of interest present in the samples are efficiently separate by liquid chromatography, while the mass spectrometry is used to identify and quantify the mycotoxins with a high sensitivity.

27. Large quantities of mycotoxins can be identified and quantified using LC-MS platforms, allowing to measure hundreds of mycotoxins simultaneously (Battliani *et al*., 2020), however, these advanced multi-mycotoxins analyses are not widely performed due to their associated high cost.

Current state of authoritative assessment and research

28. This section aims to provide a summary of the current state of authoritative assessment and research investigating the effects of mycotoxin mixtures in food and feed.

29. Several research projects and international meetings have been launched following the increasing interest of risk assessors, regulators and scientific community on the risk assessment of multiple mycotoxins. These include the 'International Conference on Food Contaminants: challenges in chemical mixtures', and a course and symposium within the 51st Congress of the European Societies of Toxicology, both delivered in 2015. The EFSA has recently published an external scientific report on Mycotoxin mixtures in food and feed: holistic, innovative, flexible risk assessment modelling approach (MYCHIF) in January 2020 (Battilani *et al*., 2020).

30. Several research groups are also working on this topic: a Portuguese project entitled 'MYCOMIX; Exploring the toxic effects of mixtures of mycotoxins in infant food and potential health impact' is led by Paula Alvito (Alvito *et al*., 2015), and the Mycotoxin and Toxigenic (MYTOX) research group is co-ordinated by Sarah De Saeger.

European Food Safety Authority (EFSA) external scientific report on mycotoxin mixtures in food and feed

31. In this report, Battilani *et al*., (2020)[7] performed an extensive literature review out across four topics relating to the investigation of mycotoxin mixtures present in food and feed. These topics were:

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 native, modified and co-occurring mycotoxins;
iii). Toxicity, toxicokinetics toxicodynamics and biomarkers relevant to humans and animals and;

[7] European Food Safety Authority external scientific MYCHIF report is available on the EFSA website.

iv). Modelling approaches, and key refence values for exposure, hazard and risk modelling.

32. The data collected from these topics 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.

33. It was observed that *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp., were the most relevant mycotoxins worldwide, this also extends to *Alternaria* spp. and *Clavicleps* spp. to a minor extent. The production of mycotoxigenic fungi is 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 native varieties as a result of fungi-host plant interaction or during processing.

34. 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 good practice at all production stages.

35. In terms of 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 fungi species identity and quantitative results. The most widely used quantification method is High Performance Liquid Chromatography (HPLC). Liquid Chromatography coupled to Mass Spectrometry (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. Furthermore, what remains important is the meaningful interpretation of the mycotoxin mixture data to their related effects in human and animal health.

36. Toxicity, toxicokinetics and toxicodynamic parameters for humans and animals were collected from the literature to build three databases in the MYCHIF platform. These are: data of toxicokinetic *in* vitro, data of toxicokinetic *in vivo*, and *in vivo* toxicity data. Limited numbers of articles on mixtures were identified in comparison to those exploring effects of single compounds. The information available only covers a limited number of combinations of mycotoxins. Toxicokinetic data were mainly reported in pigs and chickens, and rats. Toxicodynamic modelling of mycotoxin mixtures could not be performed based on the current available data.

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

38. As part of the MYCHIF project, Gkrillas *et al*., (submitted) a review paper on *in vivo* toxicokinetic data. In this report, it was highlighted that testing of all mycotoxin mixture combinations was unfeasible, as such, focus should be given to 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.

39. In terms of 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. A multi-biomarker study was defined whereby both the parent and one or more metabolite was measured. Regarding BM 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 (TEA), fusarenon-X (Fus-X,) neosolaniol (NEO), CIT, nivalenol (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.

40. In summary, it was noted that the simultaneous determination of more than one mycotoxin in human biological fluids presents a new challenge in mycotoxin biomonitoring. There are already 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 on the human bioavailability of the toxin combination, the excretion rate, and a consensus of a validated biomarker to be used in context to a multi-mycotoxin analysis.

41. It was concluded that there is still a lack of harmonisation in the experimental settings and design of biomonitoring studies, in the data collection and in the

definition of performance of fit for purpose analytical methods. These highlighted issues made it difficult 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.

42. 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 and provisional daily intake modelling methodologies (further detailed in the Exposure assessment section). Problem formulation, exposure assessment, hazard assessment and risk characterisation were completed. In brief, due to data gaps and limitations a robust risk assessment could not be performed, however, results from the component-based modelling approach calculated Margin of Exposure (MOE) values of <100 for both mixtures, indicating a potential health risk.

43. 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 risked exposure groups.

44. To conclude, the following data gaps and research recommendations were observed and/or suggested. There was still limited knowledge on the presence and co-occurrence of multiple mycotoxins, both for native mycotoxins and their modified forms, in food and feed. Available analytical methods have limitations for the routine monitoring of modified mycotoxins in food and feed. In the context of multi-mycotoxin analysis and the use of LC-MS; 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.

45. In terms of toxicity data, a limited number of articles on mixtures were identified in comparison to those only exploring the effects of single compounds. The available information only covers a very limited combination of mycotoxins and the toxicokinetic data is mainly available in livestock species (pigs and chickens), as well as rats. The modelling of toxicodynamic features of mycotoxin mixtures could not be performed based on the limited number of data available. The development of a prioritisation criteria of mycotoxin mixtures to be tested was therefore suggested as a further research priority. In addition to this, the harmonisation of methodologies and consensus guidelines for generating *in vitro* and *in vivo* toxicokinetic (TK) and toxicodynamic (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 modelling in the context of TK and TD should be further considered and explored.

46. With reference to the use of biomarkers for exposure assessment purposes; there is a need to define models in order to derive qualitative and quantitative correlation 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).

Opinions by authoritative groups on some mycotoxin combinations

47. Assessments of some binary mycotoxins 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.

Combined effects of 4,15-Diacetoxyscirpenol (4,15-DAS) with other mycotoxins T-2 and HT-2 toxins, aflatoxins, ochratoxin A and fumonisins

48. During their review of 4,15-DAS in 2018, EFSA considered the combined effects and interactions of 4,15-DAS with T-2 and HT-2 toxins, AFs, OTA and FBs (EFSA, 2018).

49. It was noted that the available data was mainly *in vivo* in poultry (Hoerr *et al*., 1981; Diaz *et al*., 1994; Kubena *et al*., 1993, 1994, 1997; Sklan *et al*., 2001; Fairchild *et al*., 2015), but one experiment was also performed in pigs (Harvey *et al*., 1991). These experiments investigated the effect of 4,15-DAS withT-2 toxin, AFB1, OTA and fusaric acid. Except for fusaric acid that reduced the effect observed with 4,15-DAS, all other experiments revealed a greater effect when 4,15-DAS was present in combination with T-2 toxin, AFB_1 and OTA. The EFSA Panel on Contaminants in the Food Chain (CONTAM) noted that because of the lack of dose–response data, it was difficult to perform a refined statistical analysis and to draw definitive conclusions concerning the nature of the combined effects.

50. A few in vitro experiments were performed to investigate the combined effect of DAS and other mycotoxins (Alassane-Kpembi *et al*., 2017) but they were not sufficient for establishing the nature of combined effects.

51. The EFSA CONTAM Panel noted that the available database describing possible effects of combined exposure to 4,15-DAS and other mycotoxins is weak and inconclusive.

Interactions between Citrinin (CIT) and ochratoxin A (OTA), and citrinin and patulin (PAT)

52. The EFSA CONTAM Panel examined available publications addressing interactions of CIT with PAT, AFs and OTA, particularly on the subject of synergism (EFSA, 2012a).

53. Data for the binary combination of CIT and OTA was the most abundant with studies from several species of animal models including dogs (Kitchen *et al*., 1977), mice (Bouslimi *et al*., 2008), poultry (Vesela *et al*., 1983; Manning *et al*., 1985; Brown *et al*., 1986), rabbits (Kumar *et al*., 2008), and rats (Siraj *et al*., 1981; Mayura *et al*., 1984). *In vitro* studies were also reviewed from various publications by Sansing *et al*., (1976), Šegvić *et al*., (2012), Heussner *et al*., (2006), and Braunberg *et al*., (1994), Bernhoft *et al*., (2004).

54. The reviewed data for CIT and AFB1 was a study carried out in poultry (chicken) by Ahamad *et al*., (2006) and an *in vitro* study by Bernhoft *et al*., (2004) for the binary combination of CIT and PAT. It was concluded that the available evidence indicated that citrinin at low doses does not exacerbate the toxic effects of other mycotoxins; the EFSA CONTAM Panel concluded that the combined effect of citrinin and OTA is, at most, additive.

Combined toxicity of fumonisins with other mycotoxins

55. The JECFA have previously considered the toxicology associated with concurrent exposure to FBs and other mycotoxin agents in 2011. Numerous *in vivo* and *in vitro* studies have shown a wide range of response suggesting antagonistic, additive (no interaction) and more-than additive (synergy) responses. It was however observed that many of the studies involve only single dose level of individual mycotoxins, and as such the Committee concluded that none of the studies were adequate for quantitative assessment of interactions (JECFA, 2011).

56. Studies by Carlson *et al*., (2001) and Gelderbloom *et al*., (2002) were noted as these documented the ability of FB_1 to promote AFB_1 hepatocarcinogenicity in trout and orally dosed pure FB_1 in rats induced precancerous lesions, respectively. The potential interaction between DNA-reactive AFB_1 and FB_1, with its potential to induce regenerative proliferation was noted as a concern by the JECFA Committee (JECFA, 2011).

57. The topic was revisited in the JECFA 2018 evaluation (Riley *et al*., 2018). The foods with the highest concentrations of AFB_1 are groundnuts, cereals (sorghum, maize, rice, and wheat), tree nuts and some spices. Foods of the highest FB_1 concentrations were maize and maize products. From international estimates of

dietary exposure, in the G05 food cluster (which includes Guatemala and Mexico) and G13 (made up of African countries), the lower bound mean AFB_1 exposures were >7 ng/kg bw/day, and the lower bound mean FB_1 exposures were >400 ng/kg bw/day. JECFA further reviewed the co-exposure for infants based on literature reports of detection of both aflatoxins and FBs in human breast milk. Infant foods are primarily cereal based and as such there can be co-exposure to AFs and FBs in the diet of infants from infant foods.

58. JECFA concluded that even though there are additive or synergistic effects observed from FB_1 and AFB_1 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. Furthermore, two prospective epidemiological studies (Magoha *et al*., 2016; Shirima *et al*., 2015), do not support the hypothesis of an interaction between AFB_1 and FB_1 in childhood stunting. JECFA concluded that there were few data available to support co-exposure as a contributing factor in human disease. However, the interaction between AFB_1 (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.

59. It was recommended that exposures to both compounds should be reduced and that emphasis on human studies should be on biomarker-based approaches.

60. JECFA further assessed the combined toxicity of FBs and DON in their 2018 evaluation. Reviewed *in vivo* studies included data from mice (Bondy *et al*., 2012; Kouadio *et al*., 2013), chickens (Antonissen *et al*., 2015) and pigs (Bracarense *et al*., 2012; Grenier *et al*, 2013). They concluded that some of the effects from co-exposure were suggestive of being additive or more than additive, however, the effect is dependent on the endpoints measured (JECFA, 2018).

Relative potencies and dose additivity of trichothecenes

61. The SCF have provided a basic summary for the relative potency and dose additivity of trichothecenes, these are provided below (SCF, 2002).

62. Although different types of trichothecenes appear to cause similar effects (T-2 toxin, HT-2 toxin, deoxynivalenol and nivalenol) at the biochemical and cellular level and there are similarities in toxic effects, there are also considerable differences in the spectrum of toxic effects *in vivo*. Large, non-systematic potency differences between these toxins are seen when different endpoints are being considered. Additionally, there are only few studies that address the combined effects of these toxins. As mentioned in numerous areas in this document, comparison of livestock

studies may pose as a challenge when comparing between toxic effects caused by naturally contaminated feed and purified extracts.

63. 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. At the time of review, the SCF were not aware of any other NIV with other trichothecene combinations 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.

64. In conjunction to this, the SCF did not support the establishment of a group TDI for all trichothecenes evaluated. Furthermore, synergism was not observed.

Mycotoxin mixtures (MYCOMIX)

65. The MYCOMIX project 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.

66. The MYCOMIX output is said to contribute to hazard identification and characterisation, as well as to exposure characterisation – and thereby contributing to risk analysis. The overview risk assessment of dietary exposure to multiple mycotoxins in Portuguese children is summarised in the next paragraphs.

67. The first study by this group was published in 2015, it aimed to perform the risk assessment of single and multiple mycotoxins present in breakfast cereals consumed by children (1-3 years old) from Lisbon, Portugal.

68. A study by Assunçao *et al*., (2018) assessed the cereal-based food products consumption by children aged from one year up to and including three years of age (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) and estimated the risk associated with the exposure to multiple mycotoxins through simultaneous consumption of different cereal-based products.

69. A total of 52 different cereal-based products marketed for children were obtained from supermarkets in Lisbon, Portugal in 2014-2015. Products included breakfast cereals (n=26), infant cereals (n=20), and biscuits (n=6). The presence of 13 mycotoxins (aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1), OTA, fumonisins

(FB_1 and FB_2), trichothecenes (DON, NIV, T-2 and HT-2 toxin) and ZEN were determined.

70. Different analytical methodologies were applied. High performance liquid chromatography with fluorescence detection was utilised to determine AFs and OTA. Trichothecenes were determined by gas chromatography coupled to mass spectrometry (GC-MS). FBs and ZEN were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS). The limit of detection (LOD) values ranged between 0.001 (for AFB_2) and 6.8 µg/kg (for T-2), and the limit of quantification (LOQ) values ranged between 0.004 (for AFB_2) and 22.3 µg/kg (for T-2). ZEN had a LOD and LOQ of 0.12 and 0.40 µg/kg, respectively.

71. Consumption data were recorded in a three-day food diary, an estimation of the exposure was evaluated, computing the intake of each mycotoxin from different food products either deterministically or probabilistically. The authors carried out risk characterisation by comparing daily intake values with the reference dose values.

72. At least one of the analysed mycotoxins was measured in forty-nine out of 52 samples (94%) (with values above the LOD). All studied mycotoxins were detected, except AFG_2, T-2 and HT-2 toxins. OTA, ZEN and DON were the most commonly detected with 65%, 48% and 44% of analysed samples revealing values above the LOD, respectively. The levels in the analysed samples were below legislative limits.

73. Analysis 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.

74. Food diary analysis revealed that ~92% of the children 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.

75. Worst-case exposure (where values < LOD = LOD) for the summed daily intake of mycotoxins present in cereal-based products (breakfast cereal, infant cereal and biscuits) are presented in **Table 1**.

Table 1 - Sum of worst-case (where values < LOD = LOD) children's daily intake of mycotoxins present in cereal-based products (breakfast cereal, infant cereal and biscuits) through 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 M_1, AFB_1; Aflatoxin B_1, AFB_2; Aflatoxin B_2, AFG_1; Aflatoxin G_1, OTA; Ochratoxin A, FB_1; Fumonisin B_1, FB_2; Fumonisin B_2, DON; Deoxynivalenol, NIV; Nivalenol, ZEN; Zearalenone.

76. 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 AFB_1. On the other hand, processed cereal-based foods (flours) presented the highest contribution for the estimated daily intakes of AFM_1, AFB_2, AFG_1, and OTA.

77. The risks derived from exposure of children to mixtures of mycotoxins in breakfast cereals were assessed, and the authors concluded that there was a potential health concern in this population group.

78. The authors recommended that a national monitoring program was carried out in view of establishing legal protective values. Additionally, further research should be conducted on obtaining toxicological data, including health consequences resulting from early-life exposures to multiple mycotoxins. Good farming and food production processes should also aim to reduce the generation of mycotoxins in crops, and decontamination of foods should take greater consideration for foods destined for children consumption.

Mycotoxin and Toxigenic moulds (MYTOX)

79. MYTOX[8] is an association research platform that strives to solve current mycotoxin problems endemic in human food and animal feed by incorporating it into a global research framework, which is based on the following four units: toxigenic

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

moulds, mycotoxins, mycotoxins and human health, and mycotoxins and animal health.

80. The mycotoxins and human health research unit includes all research projects in relation to the occurrence of mycotoxins and their effects on human health including epidemiological studies, risk assessment studies and scenario analyses. It is, however, unspecified whether there are ongoing projects on the potential risks of aggregate dietary exposure to mycotoxins.

Toxicokinetics

81. As alluded to in the MYCHIF report, a limited number of articles on mixtures were found in the literature in comparison to those examining the effects of single compounds. The information available only covers a limited number of combinations of mycotoxins. *In vivo* toxicokinetic data were mainly reported in pigs and chickens, and rats.

82. Only one study was identified which investigated the human metabolism of the *Fusarium* mycotoxins DON and ZEN using a multi-biomarker LC-MS/MS method (Warth *et al*., 2013). Urine samples from one male volunteer (60 kg) were collected and analysed following exposure to a naturally contaminated diet containing 138 µg DON and 10 µg ZEN for four days, equivalent to 2.3 µg DON/kg bw/day, and 0.2 µg ZEN/kg bw/day. In relation to their respective tolerable daily intakes, DON was 230%, whilst ZEN was at 83%.

83. A mass balance was established based on the mycotoxin intake and the concentrations of mycotoxin conjugates in the urine. The average rates of DON excretion and glucuronidation were determined to be 68 and 76%, respectively. DON-15-glucoronide was the main conjugation product, as well as DON-3 - glucuronide. The mean excretion rate of ZEN was determined to be ~9% and it was mainly present in its glucuronide form and in some cases ZEN-14-glucuronide.

84. The authors concluded that the data obtained were not necessarily valid in due to natural inter-individual variations, and so the experiment needed to be extended to a larger group of individuals.

85. A review by Gkrillas *et al*., (submitted) 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

86. This section is presented in three sections. Firstly, a review of the toxicity of single mycotoxins is provided to allow the potential grouping of compounds based on their toxic effects. Secondly, any available data on relative potencies for mycotoxin groups are discussed, and lastly, a review of the toxicity of common binary mixtures found in literature are summarised.

Review of the toxicity of single mycotoxins

87. Regulatory limits have been set for a number of mycotoxins as set in Regulation (EC) No. 1881/2006 (and its amendments)[9], however, these limits only consider the effect of single mycotoxins and do not consider their combined effects.

88. Tables have been compiled to provide a detailed yet succinct overview of all mycotoxin families previously covered in the scope of TOX/2017/30[10], their associated mycotoxins, the species of fungus that produces them, as well as their recommended health-based guidance values as set by authoritative bodies such as EFSA, JECFA, SCF (Annex B). It is hoped that the gathered data could assist in the grouping of mycotoxins based on their toxic endpoints.

Review of the toxicity of common binary mixtures

89. 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.

90. The co-occurrence of two mycotoxins in food commodities is the most studied. As such, this review focuses on binary mixtures involving the mycotoxins that are most commonly detected in cereal and cereal-based products in Europe; DON, FBs, and ZEN. Binary mixtures of AFBs were also carried out since it presents as a greater risk to humans.

91. In mixtures toxicology, there are three main different categories of interactions between mycotoxins. These are:

i). Additive – where the observed effect of the mycotoxin combination is the sum of the individual effects of the two studied toxins;

[9] Regulation (EC) No 1881/2006 on setting maximum levels for certain contaminants in foodstuffs is available on the European Commission website.
[10] TOX/2017/30 scoping paper available on the COT website.

ii). Antagonistic – where the observed effect of the mycotoxin combination is less than expected from the sum of the individual effects of the studied mycotoxins and;

iii). Synergistic – where the observed effect of the mycotoxin combination is greater than what is expected in comparison to the sum of the individual effects of the two studied mycotoxins.

92. A paper by Speijers & Speijers (2004) on combined toxic effects was one of the first reviews to assess the topic. It concluded that, at the time of writing, the tools were not fully developed to establish the type of interaction or whether there was 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).

93. Authoritative groups such as EFSA, JECFA, and SCF have provided opinions on some binary mycotoxin mixtures (refer to paragraphs 47-64).

Interactions between aflatoxins

In vitro

94. Braicu *et al*., (2010) studied the cytotoxicity of the interaction between AFB_1 and AFB_2 by *in vitro* experiments using the Methyl Thiazol Tetrazolium (MTT) cytotoxicity test[11] with three systems, namely human umbilical vein endothelial cells (hUVEC), human lung fibroblasts (hLF) and human ovarian cancer cell line (A2780 cells). The cell cultures were treated with a mixture of AFB_1 and AFB_2 at concentrations between 0 and 96 µM for 48 hours, observing a synergistic effect in hUVEC and additivity in hLF and in A2780 cells.

95. Friedman *et al*., (1997) carried out *in vitro* experiments to assess the toxicology of interaction between AFB_1 and AFB_2 in rats. They treated rat liver slices with the two mycotoxins at different concentrations (AFB_1 at 0, 120, 240 and 480 ng/ml; AFB_2 at 120 ng/ml), observing no interaction between the two mycotoxins for RNA synthesis and membrane integrity of the hepatocytes culture.

Interactions between aflatoxins and Fusarium mycotoxins

Aflatoxins and Fumonisins

96. AFB_1 and FB_1 can naturally co-occur together in cereal grains, AFB1 co-occurred with FB1 in a high-incidence area of human primary hepatocellular

[11] Methyl Thiazol Tetrazolium MTT assay is a colourimetric assay for assessing cell metabolic activity.

carcinoma in Haimen and Guangxi (Republic of China), suggesting that the mixture may be involved in the development of the disease (Ueno *et al*., 1997; Li *et al*., 2001). As such, this mycotoxin combination is one of the most highly studied binary mycotoxin mixtures.

In vitro

97. Mary *et al*., (2012) investigated the effects of exposure of AFB_1 and FB_1 (20 µM AFB_1 and 10 µM FB_1) alone or in combination using spleen mononuclear cells from Wistar rats for 48 hours.

98. The MTT assay did not show significant effects in cell viability or total cell death. The inhibitory concentration, 50% (IC_{50})[12] of individual AFB_1 and FB_1 were 166 and 230 µm, respectively, whilst the mixture had an IC_{50} of 125 µm AFB_1 and 62 µm FB_1 at 48 hours.

99. In terms of reactive oxygen species (ROS), all treatments increased total ROS and oxidation of biomolecules in a time dependent matter, with the combined exposure resulting in having the greatest effects. *Figure* 1 displays a schematic representation of the biochemical pathways involved in ROS generation induced by AFB_1 and FB_1 in SMC. Additionally, cells co-exposed were the only groups to have increase superoxide anion radical levels.

100. The authors concluded that from the results, a synergistic effect of the mixture was observed as it induced the greatest oxidative stress due to its stronger pro-oxidant action and biomolecular oxidative damage.

101. McKean *et al*., (2006a) investigated the cytotoxic effect of AFB_1 and FB_1 mixtures in hHeP-G2 and BEAS-2B cells. hHep-G2 cells were exposed to 0.13, 0.25, 0.38, 0.50, 0.75, 1.00 µM AFB_1 and 49.9, 99.8, 149.7, 199.6, 299.4, 399.2 µM FB_1, respectively for 24 hours. BEAS-2B cells were exposed to 44.4, 88.8, 133.2, 177.5, 266.3, 355.1 µM FB_1 with100 µM AFB_1, respectively for 24 hours.

102. The estimated IC_{50} for hHeP-G2 cells was 200.1 µm, the measured IC_{50} was 333 µm, and the interaction index was 0.60. As for BEAS-2B cells, the estimated IC_{50} was 277.5, the measured IC_{50} was 196.9 and the interaction index was 1.41. The results suggest a weak antagonistic effect of the mycotoxin mixture in hHep-G2 cells and an additive interaction in BEAS-2B cells.

[12] Inhibitory concentration, 50% (IC_{50}): is a measure of the potency of a substance in inhibiting a specific biological or biochemical function by 50%.

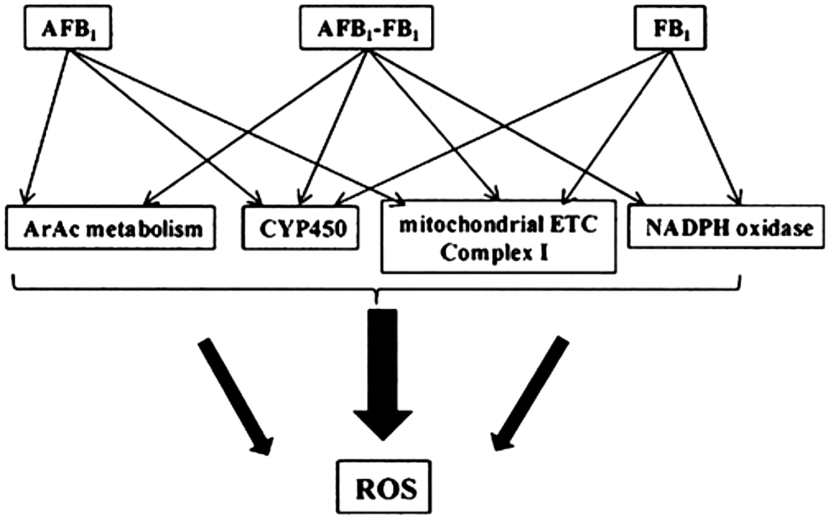

Figure 1 - shows a flowchart of the different biochemical pathways that results in reactive oxygen species production as a consequence of exposure from AFB_1-only, AFB_1 and FB_1, FB_1-only mixtures (reproduced from Mary *et al*., 2012).

In vivo

103. Qian *et al*., (2016) carried out a study to validate the co-carcinogenic effect of AFB_1 and FB_1 using a similar short-term rat model as performed by Gelderblom *et al*., (2002) (see paragraph 114 below). The main modification was the administration of mycotoxins through feeding instead of oral gavage and without partial hepatectomy[13].

104. Male F334 rats (n=62) were randomly split into five groups: (i) negative control (n=10) fed only with basal diet for 56 days, (ii) AFB_1-only treatment group (n=13) were fed with AFB_1-only containing feed (150 µg/kg diet) for the initial 14 days then followed with the basal diet for the remaining 42 days, (iii) FB_1-only treatment group (n=13) were fed with the basal diet for the first 35 days, then were fed FB_1-only containing feed (250 mg/kg diet) for an additional 21 days, (iv) the AFB_1 and FB_1 co-treatment group (n=13) were fed with AFB_1-only containing feed (150 µg/kg diet) for the initial 14 days, followed by normal basal diet for 21 days, and then switched to FB1-only containing feed (250 mg/kg diet) for an additional 21 days and, (v) the positive group (n=13) were given a single intraperitoneal injection of diethylnitrosamine (DEN)[14] (dissolved in DMSO) at 200 mg/kg body weight (50µL/100g body weight) and then maintained on the normal basal diet for 14 days

[13] Hepatectomy: is a surgical resection (removal of all or part) of the liver.
[14] Diethylnitrosamine: is a pale and yellow liquid, it is classified as a Group 2A carcinogen (probable human carcinogen) by the World Health Organisation.

followed by feeding with the 2-acetylaminofluorene (2-AAF)[15] containing diet (150 µg/kg diet) for 21 days.

105. Serum biochemistry, histopathological alterations, and liver placental glutathione S-transferase (GST-P+) foci formation were monitored. Sequential exposure resulted in increased dysplasia, apoptosis and foci of altered hepatocytes in co-exposed groups. Sequential exposure was observed to increase liver GTP-P+ foci number by 7.3- and 12.9-fold, when compared to AFB_1 and FB_1 treated groups alone.

106. Theumer *et al.*, (2008) investigated the probable synergism of FB_1 and AFB_1 in the induction of hepatocyte apoptosis in male Wistar rats (n=24; 6/group). The treatment groups were control (basal diet only), diet containing 100 ppm FB_1, diet containing 40 ppb AFB_1, and a diet consisting of 40 ppb and 100 ppm of AFB_1 and FB_1, respectively. Each group were fed for 90 days. Histopathological analyses of lung, liver, kidney and small intestine biopsies revealed that lesions were observed for all organs in the group treated with the combined mycotoxin diet. The co-exposure to AFB_1 and FB_1 induced higher occurrences of apoptosis and mitotic hepatocytes, in comparison to the control and individual administration groups. The sphinganine (Sa) and sphingosine (So)[16] concentrations, and Sa/So ratios in urine, serum, liver and kidney samples were also measured. Of all the biological samples analysed, the liver and kidney homogenates presented the greatest increase in Sa and the Sa/So ratio when compared with the control.

107. Orsi *et al.*, (2007) determine the effects of oral administration of AFB1 and FB1 (>95% purity) alone and in combination to rabbits by clinical, pathological, biochemical and sphingolipid analyses. Male white New Zealand rabbits (n=24; 6 per group) were treated with oil-based AFB_1 suspension and sterile saline-based FB_1 solution for 21 days by oral gavage in the following dose groups: control (no mycotoxin content), AFB1-only treatment group (30 µg/kg bw/day), FB1-only treatment group (1.5 mg/kg bw/day), co-exposed group (combination of AFB_1 (30 µg/kg bw day) and purified FB_1 (1.5 mg/kg bw day).

108. Results from clinical observations showed that AFB1-only and co-exposed treatment groups showed clinical signs of intoxication including apathy, anorexia, lethargy, ruffled fur and loss of body weight. Absolute and organ (liver and kidney) weights were also found to significantly decrease in the co-exposed group. Mortality (n=3/6) was also recorded in the co-exposed group at days 14, 15- and 18-days post-administration.

[15] 2-acetylaminofluorene (2-AAF): is a carcinogenic and mutagenic derivative of fluorene.
[16] Sphinganine (Sa) and Sphingosine (So): comprises the backbone of all sphingolipids. They and their phosphorylated derivatives are also important second messengers involved in functions such as cell growth, differentiation, and apoptosis

109. In terms of biochemical parameters, AST and ALT levels were 4 and 20 times higher respectively in the co-exposed group compared to the controls. The AP and GGT levels observed were suggestive of an additive effect on AP and a synergistic effect on GTT, indicating hepatic and/or biliary injury. High urea and creatinine levels (155 and 4 mg/dL, range of 33-154 and 1-4 mg/dL, respectively) were also observed from the co-exposed group, reflecting renal injury and a synergistic interaction. The Sa/So ratios observed in urine (4.67), serum (1.14) and liver (0.82) were higher than other treated groups.

110. As for histopathological results, Orsi *et al.*, (2007) observed an increase in gallbladder volume and apparent thickening of the bile in rabbits in the AFB1-only treated group. FB1 exposure resulted in the degeneration of the epithelium of the proximal tubules. Both hepatic and kidney alterations were more marked in the co-exposed group. The authors concluded that the liver and kidney were the target organs in FB1 induced rabbit toxicosis and that the combined administration of AFB1 and FB1 resulted in synergistic toxic effects in the liver and kidneys.

111. McKean *et al.*, (2006a) also investigated the combinative toxicity of AFB_1 and FB_1 (both >98% purity) in animals (male Fischer 344 rats and mosquitofish (*Gambusia affinis*)). The potency of the mixture was gauged through the determination of the interaction index metric[17]. Male Fischer rats (n=56; 8/group) were split into 7 groups. One group was given only the solvent carrier (DMSO) as a control, other were treated with AFB_1 at concentrations of 0.34, 0.68, 1.02, 1.36, 2.03, 2.71 mg/kg bw and 25 mg/kg bw of FB_1. The study was completed over 14 days.

112. Mortality was observed in groups treated with 1.02 and 1.36 mg/kg bw AFB_1 with 25 mg/kg bw FB_1 (n=2/8). Additionally, all animals in the highest dose groups (2.03 and 2.71 mg/kg bw AFB_1 with 25 mg/kg bw FB_1) died (n=8/8). The estimated lethal dose, 50% (LD_{50})[18] as a result of co-exposure to AFB_1 and FB_1 was 2.71 mg/kg bw, the measured LD_{50} was 1.37 mg/kg bw and the interaction index was 1.98. The authors concluded that an additive effect in the *in vivo* rat experiments.

113. As for the mosquitofish, they were divided in 7 groups, each containing 12 fish (half male and half female), and treated with AFB_1 at 85.1, 170.3, 255.4, 340.5, 681.0 µg/L and FB1 at 580, 1160, 1740, 2320, 4640 µg/L for 5 days. Mortality was observed in all treated groups at in a dose dependent manner. At the highest combined dose all animals died (n=12), whilst the lowest combined dose ranges

[17] The interaction index is the ratio of estimated $LD_{50}/LC_{50}/IC_{50}$ divided by measured $LD_{50}/LC_{50}/IC_{50}$.
[18] Lethal dose, 50 (LD_{50}) is the dose required to kill half the members of a tested population after a specified test duration.

(AFB_1 at 85.1, 170.3 and FB_1 580, 1160 µg/mL, respectively) had 3 deaths. The measured lethal concentration, 50% (LC_{50})[19] was 1,342.7 µg/mL, and the interaction index was 1.98. The authors concluded that an additive effect in the *in vivo* fish studies.

114. Gelderblom *et al*., (2002) investigated the cancer potency of AFB_1 and FB_1 in male Fischer rats (n=5-8 animals/group). Two protocols were utilised. Firstly, the potency of AFB_1 and FB_1 were compared with DEN using a resistance hepatocyte model with 2-AAF/partial hepatectomy as the promoting stimuli. Three groups were treated separately for the initiation step. One group was treated with a single intraperitoneal dose of 200 mg DEN /kg, the other a daily oral gavage administration of 17 µg AFB_1/kg bw for 14 days (totalling to 0.25 mg AFB_1/kg), and lastly, dietary exposure of 250 mg FB1/kg for 21 days. The promotion step was carried out 3 weeks after initiation. In the case of the 2-AAF/PH promoting regimen the rats received an oral gavage dosage of 2-AFF (20 mg/kg bw) on three consecutive days followed by partial hepatectomy on day four. Control groups were 2-AAF/PH and PH groups for cancer promotion and carrier solutions of dimethyl sulfoxide (DMSO) or DMSO with water (1:1) as initiation controls.

115. The second protocol monitored the combined effects of AFB_1 and FB_1. Rats received oral gavage administration of 17 µg AFB_1/kg bw for 14 days (totalling 0.25 mg AFB_1/kg), followed by a 3-week latent period, they were then exposed to 250 mg FB1/kg in the diet for 21 days. The control groups received the carrier (dimethyl sulfoxide: water) at 0.2 mL/100 g bw/day for 14 days, 17 µg AFB_1/kg bw oral gavage treatment for 14 days and dietary exposure to 250 mg FB1/kg for 21 days.

116. The authors estimated an effective dosage level (EDL) for cancer initiation by FB_1 treatment (*via* gavage) over a period of 14 days and 21 day feeding study in male Fischer rats. This was $0.39 < EDL < 0.83$ and $0.7 < EDL < 1.5$ mg FB_1/100g bw per day, respectively. FB_1 was described to be between 200 and 400 times less potent as a cancer initiator than AFB_1.

117. Despite the 3-week latency period, AFB_1 and FB_1 were observed to act synergistically with respect to cancer initiation. The authors observed increased liver weight, cirrhosis in the liver, apoptotic cellular bodies, and the increased number and size of hepatocyte nodules and foci. The authors concluded that AFB_1 may promote cancer potency of FB_1.

[19] Lethal concentration, 50% is the concentration required to kill half the members of a tested population after a specified test duration.

Aflatoxins and Deoxynivalenol

In vitro

118. Sun *et al*., (2015) investigated the effect of the mixture AFB_1 and DON on Buffalo rat liver 3A (BRL 3A) cells. The cells were treated with 22, 24, 29, 34, 36 μM AFB_1 and 0.2, 1, 3, 5, 5.8 μM DON, respectively for 24, 48 and 72 hours. The mixtures of AFB_1 and DON were observed to have a synergetic effect on BRL 3A cells by decreasing cellular viability and the promotion of apoptosis as a result of ROS production. The effects were mediated by the upregulation of apoptotic genes (Hsp70, p53, Bax, caspase-3 and caspase-8) and the downregulation of the anti-apoptotic gene Bcl-2, which suggested a synergistic effect.

119. Lei *et al.* (2013) studied the cytotoxicity of the interaction between AFB_1 and DON in cell line porcine kidney PK15. Cells were exposed to varying concentrations of 1 μM AFB_1 and 1, 2, 4 μM DON or 1, 5, 10 μM AFB_1 and 1 μM DON for 24 hours. ROS production was utilised as a measure for cellular stress and induction of apoptosis. Synergistic effects were observed in a concentration-dependant manner at high doses of AFB_1 (1 μM DON and 5 or 10 μM AFB_1).

120. Šmerák *et al.* (2001) studied the mutagenic activity of AFB_1 in combination with DON by using the Ames test (*Salmonella typhimurium*; TA98 and TA100 with S9 metabolic activation). The *in vitro* experiment was carried using varying concentrations of AFB_1 at 1.0, 0.5, 0.25 and 0.8 μg and DON at 8.0, 4.0, 2.0, and 0.1 μg/dish, respectively. The combination AFB1 and DON induced significantly greater number of revertants in both test strains, which suggested a synergistic effect.

Aflatoxins and Zeralenone

In vitro

121. Sun *et al*., (2015) additionally investigated the combined effect of AFB_1 and ZEN on BRL 3A rat liver cells. The cells were treated with 22, 24, 29, 34, 36 μM AFB_1 and 78, 82, 92, 102, 106 μM ZEN, respectively for 24, 48 and 72 hours. The mixture showed a synergetic effect on BRL 3A cells, decreasing the viability of cells by inducing intracellular ROS production and promoting apoptosis in the BRL 3A cells.

122. Lei *et al.*, (2013) further studied the effects of AFB1 and ZEN on PK15 cells. Cells were exposure to 10 μM ZEN and 1, 5, 10 μM AFB1 or 10, 20, 40 μM ZEN and 1 μM AFB_1. The authors observed that low doses of AFB_1 (1 μM) lead to antagonistic effect in combination with ZEN, while the synergistic effect was observed at higher

doses (5 and 10 μM AFB_1). The same synergistic effect for the cytotoxicity of the combination between AFB_1 and DON was observed in the same paper by

Aflatoxins and T-2 toxin

In vitro

123. McKean *et al*., (2006b) further investigated the cytotoxic effect of AFB1 and T-2 toxin mixtures in HepG2 and BEAS-2B cells. HepG2 cells were exposed to 125, 250, 375, 500, 750 nM AFB_1 and 123, 245, 368, 490, 735 nM T-2 toxin, respectively for 24 hours. BEAS-2B cells were exposed to 2, 4, 8, 12, 16, 24 nM T-2 toxin with 100 μM AFB_1, respectively for 24 hours.

124. The reported IC_{50} for HepG2 cells was 0.99 μm, the measured IC_{50} was 1.06 μm, and the interaction index was 0.93. As for BEAS-2B cells, the estimated IC_{50} was 32 nM, the measured IC_{50} was 3 and the interaction index was 9.69. The results suggest a weak antagonistic effect of the mycotoxin mixture in HepG2 cells and an additive interaction in BEAS-2B cells.

In vivo

125. McKean *et al*., (2006b) studied the combinative toxicity of AFB_1 and T-2 toxin in animals (Fischer 344 rats and mosquitofish). The potency of the mixture was gauged through the determination of the interaction index metric. Male Fischer rats (n=70; 10/group) were split into 7 groups. One group was given only the solvent carrier (DMSO) as a control, other were treated with AFB_1 at concentrations of 0.34, 0.68, 1.02, 1.36, 2.03, 2.71 mg/kg bw and 0.46, 0.93, 1.39 1.86, 2.79, 3.17 mg/kg bw of T-2 toxin. The study was completed over 14 days.

126. Mortality was observed in groups treated with 0.68, 1.02 mg/kg bw AFB_1 and 0.93, 1.39 mg/kg bw T-2 toxin, respectively (n=1/10). Additionally, 7/10 animals died in the group treated with 1.36 mg/kg bw AFB_1 and 1.86 mg/kg bw T-2 toxin. Mortality was further observed in the highest dose groups (2.03 and 2.71 mg/kg bw AFB_1 with 25 mg/kg bw FB_1) were all animals died (n=10/10; each group)

127. The estimated LD_{50} as a result of co-exposure to AFB_1 and T-2 toxin was 3.22 mg/kg bw, the measured LD_{50} was 2.83 mg/kg bw and the interaction index was 1.13. The authors concluded that an additive effect.

128. The mosquitofish were divided in 7 groups, each containing 12 fish (half male and half female), and treated with AFB_1 at 85.1, 170.3, 255.4, 340.5, 510.9, 681.0 μg/L and T-2 toxin at 18.4, 36.8, 55.1, 73.5, 110.4 and 147.0 μg/L for 5 days.

129. Mortality was observed in all treated groups at in a dose dependent manner. At the highest combined dose all animals died (n=12), whilst in the lowest combined dose groups (AFB_1 at 85.1, and T-2 toxin at 18.4 µg/mL) there were 2 deaths. The measured lethal concentration, 50% (LC_{50})[20] was 234 µg/mL, and the interaction index was 1.77. The authors concluded that there was an additive effect in the *in vivo* fish studies.

130. Šmerák *et al*. (2001) further investigated the mutagenic activity of AFB_1 and T-2 toxin using the Ames test (*Salmonella typhimurium*; TA98 and TA100 with S9 metabolic activation). The *in vitro* experiment was carried out with varying concentrations of AFB_1 and T-2 toxin at 1.0, 0.5, 0.25 and 0.1 µg each/dish, respectively. The combination AFB1 and T-2 toxin induced significantly greater number of revertants in both test strains, which suggested a synergistic effect.

Interactions between Aflatoxins and Ochratoxin-A

In vitro

131. Corcuera *et al.*, (2011) investigated the genotoxic *in vitro* effects of AFB1 and OTA in HepG2 cells *via* a modified Comet assay[21] (restriction of endo III and FPG enzymes). Cells were exposed to OTA at concentration ranges of 50–800 µM and 100 µM or 150 µM AFB_1 for 3h (with/without metabolic activation) or 1–200 µM OTA and 100 µM or 150 µM AFB_1 for 24 hours (with/without metabolic activation).

132. No significant cytotoxicity was observed with any treatments after 3 hours exposure; however, at 24 hours cytotoxicity was observed. The calculated IC_{50} for OTA and 100 µm AFB_1 exposure groups was 100 µm, whilst the IC50 for groups exposed to OTA and 150 µm AFB_1 was 200 µm. These results suggested an additive effect. With regards to the observed genotoxic effects, combined treatments showed a significant decrease in DNA damage when compared to AFB_1 only treated groups. Conversely, ROS levels increased. These results suggest an antagonistic effect. The authors suggested that the different effects for the two endpoints are due to the competition of AFB_1 and OTA for the same cytochrome P450 enzymes.

133. Golli-Bennour *et al.*, (2010) investigated the combinative cytotoxicity and genotoxicity of AFB_1 and OTA in monkey kidney vero cells. To investigate the cytotoxic potential of the mixture; cells were exposed to mixtures of AFB1 and OTA at 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µM each, respectively for 24 hours and assessed *via* the MTT assay. To investigate the genotoxic potential of the mixture;

[20] Lethal concentration, 50% is the concentration required to kill half the members of a tested population after a specified test duration.

[21] Comet assay: is a single-cell gel electrophoresis method for detecting DNA strand breaks (either single or double) at the individual cell level.

cells were exposed to mixtures for AFB_1 and OTA at 0, 1, 5, 10, 20 and 40 µM each, respectively for 24 hours and the amount of DNA fragmentation was quantified by Western blots of p53 and Bcl-2 expression.

134. The experiments resulted in an IC_{50} of 27 µM (compared to acute IC_{50} values of 30 µM AFB_1 and 35 µM OTA, in isolation). The interaction index value was 1.2, which suggested an additive effect for both endpoints.

135. Sedmíková *et al.*, (2001) studied the mutagenic effect of the combination of AFB_1 and OTA *via* Ames test (*Salmonella typhimurium*; TA98 and TA100 with/without metabolic activation). Plates (n=6) were treated with 0.25, 0.5, 1 and 2 µg AFB1 with the combination of 0.5, 1, 2, and 4 µg OTA, respectively.

136. Plates exposed to the combination of mycotoxins induced a statistically significant increase in the number of revertants at all concentrations in both *S. typhimurium* strains with metabolic activation. A synergistic effect was concluded by the authors and it was found that OTA could increase the mutagenic effect of AFB_1; where OTA inhibits translation *via* ROS production, and AFB_1 inhibits transcription by forming AFB_1 adducts.

In vivo

137. Abdel-Wahhab *et al.*, (2015) investigated the hepatotoxic and nephrotoxic effects of AFB_1 and OTA in male Sprague Dawley rats (n=40/10 per group). One group served as a control, one with AFB_1 alone (80 µg/kg bw), one with OTA alone (100 µg/kg bw), and the last group with 80 µg AFB_1/kg bw and 100 µg OTA/kg bw. All administration was carried out *via* oral gavage, the carrier was corn oil and the duration of exposure was 8 weeks. Blood and tissue samples were collected for biochemical and histological analysis.

138. Results from liver function tests indicated a synergistic effect in the combined mycotoxin exposure group. Statistically significant increases were observed for ALT, AST, ALP, and γ-Glutamyl Transpeptidase at 20, 16, 17, 1,380 U/L, respectively compared to the control, AFB_1- and OTA-only exposed groups with values ranging at 10-16, 7-15, 1-15 and 890-1,050 U/L, respectively for the same parameters.
The same observation was observed on the kidney function tests. Statistically significant increases were recorded for uric acid, creatinine, and urea at 12 and 18 mg/dL, and 92 g/dL, respectively compared to the AFB_1- and OTA-only exposed groups with values ranging at 3-10 and 5-15 mg/dL, and 48-82 g/dL, respectively for the same parameters.

139. Markers of oxidative stress (nitric oxide) and tumours (malondialdehyde and α-fetoprotein) for the combined exposure group were also statistically significant at

320 µmol/L, 5 nmol/g tissue and 6.28 ng/mL, respectively compared to the control, AFB_1- and OTA-only exposed groups with values ranging at 160-240 µmol/L, 0.70-4 nmol/g and 2.70-5 ng/mL, respectively for the same parameters.

140. Histological changes in liver and kidney tissues were marked when compared to other treatment groups. With regards to the liver, disorganisation of hepatic cords, vascular destruction, bile duct proliferation and aggregation of inflammatory cells were observed; a marked increase in tubular necrosis, vacuolation and obliteration was also seen.

141. Based on the results, the authors concluded that the combined exposure of AFB_1 and OTA resulted in synergistic toxic effects.

142. Wangikar *et al.*, (2004a) (2004b) studied the teratogenic effects of AFB_1 and OTA, alone or in combination in pregnant Wistar rats (n=140/10 per group). Mycotoxins were administered by oral gavage at different doses of OTA (0, 0.125, 0.25, 0.50 and 0.75 mg/kg), AFB1 (0, 0.125, 0.25, 0.50 and 1 mg/kg) and a combination of OTA and AFB1 (0.125 + 0.125, 0.25 + 0.50 and 0.50 + 0.25 mg/kg, respectively) in corn oil on days 6-15 of gestation. Clinical observations were recorded daily until day 20 of gestation when all the dams were sacrificed.

143. The clinical signs observed in the dams of the combination groups were diarrhoea, polyuria, melena, lacrimation, anorexia and increased water intake. These signs were more severe in the high AFB1 (0.25 + 0.50 mg/kg) and high OTA (0.5 + 0.25 mg/kg) dosed groups. In these groups, one rat each aborted. Mortality was observed in the low, high AFB_1 and high OTA combination groups (n=1 death/treatment group) on gestation days 16, 13, and 12 respectively. The body weight gains in the high treatment group significantly decreased during and post-dosing when compared to the control groups. A non-significant increase in the percentage of implants resorbed and a decrease in the percent of live foetuses relative to controls in both the high AFB_1 and the high OTA combination groups. The percent of dead foetuses was only significantly observed in the high OTA combination group.

144. In terms of adverse effects in the foetus, foetal weights and crown to rump lengths in all the combination dose groups; did not significantly differ from the control group. Gross abnormalities including gastroschisis[22], syndactyly[23], and micrognathia[24] were observed. Skeletal anomalies such as fusion of the ribs and

[22] Gastroschisis: is a birth defect in which the foetuses' intestines extend outside of the abdomen through a hole next to the umbilicus.
[23] Syndactyly: is a condition wherein two or more digits are fused together.
[24] Micrognathia: is a condition in which the lower jaw is undersized, it may interfere with feeding and breathing.

unossified sternebrae, as well as visceral anomalies such as hydrocephalus and cardiac defects of the valves and ventricular chambers were also recorded.
The authors observed that there is some interaction between these mycotoxins that resulted in reduced teratogenic activity of OTA in the presence of AFB_1, suggesting an antagonistic effect.

Summary of AFB1 and interactions with other mycotoxins

145. Various studies of AFB1 and interactions with other mycotoxins (AFB_2, FB_1, DON, ZEN, T-2 toxin, and OTA) utilising different methodologies and assessment of different endpoints have been summarised above.

146. Investigating hepatotoxicity as endpoint, the combination of AFB_1 and FB_1 resulted in synergistic effect in rats (Gelderbloom *et al*., (2002); Theumer *et al*., (2008); Qian *et al*., (2016)) and in rabbits (Orsi *et al*., 2007).

147. A summary table is presented in the next page to provide a highlight overview of the reviewed literature (*Table 2*).

Table 2 - 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	Additive and antagonistic	McKean *et al*., (2006a)
	In vivo	Fischer 344 rats & mosquitofish	Acute	Synergistic	McKean *et al*., (2006a)
	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)
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 & mosquitofish	Acute	Additive	
AFB_1 + OTA	*In vitro*	Ames test	Mutagenicity	Synergistic	Sedmíková *et al*., (2001)
	In vitro	Monkey kidney vero cells	Cytotoxicity 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.

Interactions between ochratoxin-A and FB_1

In vitro

148. Šegvić Klarić *et al*., (2007) studied the cytotoxic effect of OTA and FB_1 in combination on porcine kidney epithelial cells (PK15) using the trypan blue exclusion assay[25]. Cells were exposed to 0.05, 0.5 and 5 µg/mL of each mycotoxin, respectively for 24 and 48 hours. The combination of the two mycotoxins, each given in the concentrations of 0.5 µg/mL or 5 µg/mL, significantly reduced viable cells (~20% and 10%, respectively). The authors concluded that the combined treatment resulted in an overall additive effect especially after 24 hours, however, synergistic as well as antagonistic interactions could not be excluded depending on the toxin concentration and the duration of exposure.

149. Mwanza *et* al., (2006) investigated the cytotoxicity of OTA and FB_1 in human mononuclear cells and pig lymphocytes using the MTT cytotoxicity assay. Cells were exposed to 5 µg/ml each of OTA and FB_1 or 20 µg/ml each of OTA and FB_1, (dissolved in 0.1% DMSO and phosphate buffer saline) for 48, 72 and 96 hours. After 24 hours, the cell viability of pig lymphocytes decreased to 50 and 60% and further decreased to 70 and 80% after 96 hours of the combined mycotoxin exposure at the low and high dose groups, respectively. For human lymphocytes a lower but significant decrease of cell viability of 47.7 (24 h) to 33.5% (96 h) at 5 µg/ml exposure, and 53.8 to 69.6% at 20 µg/ml (after 24 and 96 h, respectively) as compared to results obtained with pig cells. The results indicated a synergistic effect in both human and pig lymphocytes, with pig lymphocytes showing a greater sensitivity.

150. Creppy *et al*., (2004) studied the cytotoxicity of the combination of OTA and FB_1 in three different cell lines; rat C6 glioma, human Caco-2 and Vero green monkey kidney cells using the neutral red assay. Cellular cultures were exposed to test compounds over a range of concentrations (0-100 µm) and were assessed after 48 hours post-exposure. A reduction of cell viability was observed in all cell lines; decreases of up to 55%, 71% and 86% being recorded for C6 glioma, Caco-2 cells and Vero cells, respectively when compared to controls. The authors concluded that this was a synergistic effect.

[25] Trypan Blue exclusion assay is used to determine the number of viable cells present in a cell suspension. It is based on a principle that live cells possess intact cell membranes to exclude certain dyes such as trypan blue whereas dead cells do not.

In vivo

151. Domijan *et al*., (2006) investigated the genotoxicity of the combination OTA and FB_1 in male Wistar rats (n=72/6 animals per group) by assessing the DNA damage in kidney cells using the standard alkaline comet assay and the Fpg-modified comet assay.

152. Three groups were dosed with OTA at 5, 0.05 and 0.5 mg/kg bw, respectively for 15 days. Another three groups were dosed with FB1 at 200 ng/kg bw, 0.05 and 0.5 mg/kg bw for 5 days. A further three groups of animals were treated with OTA for 15 days and with FB1 for the last 5 days of the OTA treatment. The doses were 5 ng OTA/kg bw and 200 ng FB_1/kg bw for the first group, 5 ng OTA/kg bw and 0.05 mg FB_1/kg bw for the second group, and 0.05 mg OTA/kg bw and 0.05 mg FB_1/kg bw for the third group.

153. No mortalities were observed but all animals gained weight. The mean tail lengths, intensity and Olive tail moment (OTM)[26] were significantly higher than those compared to the control and single mycotoxin only exposed groups. The combination of OTA and FB_1 treatment showed a synergistic increase in the tail intensity and OTM in kidney cells, even at doses that correspond to the daily human exposure in Europe (based on the WHO estimated daily intake of 5 and 200 ng/kg bw of OTA and FB_1, respectively.

Interactions between ochratoxin-A and zearalenone

In vitro

154. Wang *et al.*, (2014) evaluated the cytotoxicity of OTA, ZEN and α-zearalenol (α-ZOL), alone or in combination in human HepG2 cells using the MTT assay and isobologram analysis[27]. Cells were treated with nine dilutions of each individual mycotoxin, OTA (from 0.2 to 20 μM), ZEN (from 1 to 100 μM) and α-ZOL (from 0.5 to 50 μM) and were exposed for 24, 48 and 72 hours, with a fixed constant ratio (OTA:ZEN = 0.2:1, OTA:α-ZOL = 0.4:1, ZEN:ZOL = 2:1, OTA:ZEN:α-ZOL = 0.4:2:1).

155. The proliferation of HepG2 cells decreased with the increase in of mycotoxin concentrations and exposure time in all experimental groups. In terms of isobologram analysis, the combination index values mostly reported varying signs of antagonism (from moderate to very strong). The combined effect of OTA and ZEN, OTA and α-ZOL, were overall showed strong and common antagonism, respectively.

[26] Olive tail moment (OTM) is considered to be particularly useful in describing heterogeneity within a cell population, as it can pick up variations in DNA distribution within the tail.
[27] Isobologram is a type of method where the potency ratio is defined as the dose ratio of two compounds when the same effect is achieved.

Whilst combination index values for ZEN and α-ZOL at the recorded $IC_{50\text{-}90}$ values at 48 hours showed nearly additive to moderate synergism.

156. Li *et al*., (2014) evaluated the cytotoxicity of OTA and ZEN, alone or in combination in human HepG2 and murine ovarian granular (KK-1) cells using a cell counting kit and an ROS production assay. HepG2 cells were exposed to varying concentrations of OTA at 6.61, 16.30, 24.66 and 37.30 μM and ZEN at 1.12, 7.36, 17.44 and 41.28 μM, whilst in KK-1 cells; OTA was added at 0.53, 0.80, 1.44 and 3.61 μM and ZEN at 9.98, 11.84, 15.50 and 24.40 μM for 24 hours. The gathered data were analysed using two mathematical models based on the concepts of concentration addition (CA) and independent addition (IA).

157. The observed EC_{50} value for HepG2 and KK-1 cells were 82.62 and 79.72 μm, respectively. Based on the calculated mediation deviation ratios, the IA model was deemed more accurate by the authors. Furthermore, mixture of OTA and ZEN showed additive effects, irrespective of the regression models used.

Interactions between ochratoxin-A and citrinin

In vitro

158. Šegvić Klarić *et al.*, (2012) investigate the role of calcium signalling in genotoxicity, apoptosis and necrosis evoked by OTA and CIT, alone or in combination in porcine kidney PK15 cells. The cytotoxic potential of each mycotoxin was further assessed by MTT and trypan blue assays. To determine the IC_{50} for both toxins cells were treated with OTA at concentrations of 2, 6, 10, 18, 26, 34, and 46 μM, as well as concentrations of CIT at 10, 30, 50, 70, 90, 110, and 130 μM for 24 hours. In order to evaluate the number of apoptotic cells, micronuclei, nuclear buds, and nucleoplasmic bridges cells were treated with concentrations of OTA-alone (6 and 10 μM), CTN alone (30 and 50 μM) or their combinations for 12 and 24 hours.

159. MTT and trypan blue results demonstrated that CIT (IC_{50} values of 73.5 and 75.4 μM, respectively) was less toxic than OTA (IC_{50} values of 14 and 75.4 μM, respectively). Combined treatment induced a dose depended increase in cytosolic calcium levels, however, this did not provoke an additional increase in calcium signals. Combined exposure results in apoptotic and necrotic synergism, while genotoxic effects of OTA and CIT were notes as antagonistic or additive.

160. Bouslimi *et al*., (2008) investigated the cytotoxicity and production of ROS of OTA and CIT, alone or in combination in Vero monkey kidney cells using three different cell viability assays MTT, neutral red and trypan blue. The role of oxidative

stress was assessed by measuring the malondialdehyde (MDA) level[28] and the expression of the heat shock protein (Hsp 70). To evaluate the cytotoxicity, cells were treated with increasing concentrations of OTA, ranging from 0-50 µM, or CIT at 0-250 µM, or OTA and CIT simultaneously at 0-50 µM for 48 hours. To assess the extent of lipid peroxidation, cells were exposed to OTA (12.5 and 25 µM), CIT (60 and 120 µM), and OTA (12.5 and 25 µM) combined to a fixed concentration of CIT (60 µM) for 12 hours. To measure the levels of Hsp70, cells were exposed to increasing doses of OTA (0-50 µM), CIT (0-100 µM), and a combination of the two mycotoxins OTA (0.05-1 µM) and CIT at a fixed concentration of 0.75 µM for 48 hours.

161. The observed IC_{50} values for OTA-alone, CIT-alone and combined treatment groups were 37, 220, and 24 µM for the MTT assay, respectively; 12, 175, and 5.8 µM for the neutral red assay, respectively; and 11, 160 and 8.5 µM for the trypan blue assay. The combination index for each method was 1.7, 1.6 and 1.7, which suggests a synergistic effect.

162. In terms of the induction of MDA and Hsp70 expression, there was a significant increase in the levels of both proteins in the combined mycotoxin exposure group. The authors concluded that OTA and CIT combination effects are of a synergistic nature.

163. Bernhoft *et al.*, (2004) investigated the immunotoxic effects of OTA and CIT, alone or in combination in purified lymphocytes from six piglets (Norwegian Landrace). Cells were exposed to mycotoxins at different concentrations OTA-alone (0.09, 0.18, 0.36, 0.54, 0.72, 0.9 mg/L), CIT-alone (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg/L) or a combination of the two mycotoxin concentrations.

164. From dose-response curves, the combination of OTA and CIT decreased cellular proliferation in a dose dependent a manner. Calculated IC_{20} values suggest that OTA and CIT would exert a synergistic effect.

Summary of OTA and interactions with other mycotoxins

165. Various studies of OTA and interactions with other mycotoxins (FB_1, ZEN, and CIT) utilising different methodologies and assessment of different endpoints have been summarised above. It should be highlighted that most studies were carried out using *in vitro* test methods.

166. A summary table is presented in the next page to provide a highlight overview of the reviewed literature (*Table 3*).

[28] MDA level analysis was utilised in this context to assess levels of lipid peroxidation.

Table 3 - Provides a highlight summary of the observed combinative effects of ochratoxin A (OTA) with fumonisin B1 (FB1), zearalenone (ZEN), and citrinin (CIT) 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	Porcine kidney epithelial cells (PK15)	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*	HepG2 cells	Cytotoxicity	Antagonistic	Wang *et al*., (2014)
	In vitro	HepG2 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; FB_1 = Fumonisin B_1; ZEN = Zearalenone; CIT = Citrinin; PK-15 = Porcine kidney 15 epithelial cells; HepG2 = human hepatoma cells G2; KK-1 = murine ovarian granular cells.

Interactions between Fusarium mycotoxins

Zearalenone and fumonisins B_1

In vitro

167. Kouadio *et al*., (2007) studied the cytotoxicity, lipid peroxidation, DNA synthesis, and DNA fragmentation of ZEN and DON in human Caco-2 cells. Cells were exposed to different concentrations of ZEN at 5, 10, 20 µM and 10 µM FB1 (alone or in combination) for 72 hours in cytotoxicity tests. Whilst for DNA synthesis, DNA fragmentation and lipid peroxidation, ZEN and FB_1 were added to cells at 10 µM each (alone or in combination) for 24 hours.

168. Cell viability was assessed using the neutral red assay. ZEN-only exposed cells were statistically more significant in reducing cell viability at all tested concentrations than the control and FB_1-only exposed groups, ranging from ~20-60% cell viability reduction. In terms of the combined treated groups, ZEN and FB_1 at

concentrations of 20 µM and 10 µM, respectively was observed to be less potent in reducing cell viability (~36%). The results were described as antagonistic.

169. Lipid peroxidation was measured by MDA production. The combined group induced a greater production of MDA in Caco-2 cells when compared to the control and ZEN-only and FB1-only treated groups at ~680 nmoles MDA/mg protein compared to ~390-600 nmoles MDA/mg protein. The results were described as “more than additive”.

170. In DNA synthesis, ZEN and FB_1 inhibited only 35%, as compared to their individual inhibitory potential of 70 and 45%, respectively. The observed effect was described to less than additive.

171. DNA fragmentation was evaluated by agarose gel electrophoresis. An increase in DNA fragmentation was observed as evidenced by the appearance of DNA ladders. The combined exposure groups shifted the fragment sizing towards 250 base pairs. FB1-only was observed to cause the greatest effect on DNA fragmentation followed by ZEN and FB_1.

Interactions between deoxynivalenol and other mycotoxins

In vitro

172. Yang *et al.*, (2017) assessed the individual or combined toxicological effects to multiple DON-family mycotoxins, this included: DON, NIV and their acetyl derivates 3-acetyldeoxynivalenol (3-AcDON), 15-AcDON, deoxynivalenol-3-glucoside (D3g), and Fusarenon-X (Fus-X) on the human gastric epithelial (GES-1) cells. NIV was observed to be the most toxic out of the tested mycotoxins followed by Fus-X, for individual toxic effects at 0 to 3 ppm (cell viability decreased in a dose-dependent manner). For combined toxicological effects, Fus-X + NIV at 2 ppm each decreased cell viability by up to 30%, whilst Fus-X + 15-ADON (2 ppm and 6 ppm, respectively) exhibited the least toxic effect. The former mycotoxin combination resulted in a complete synergistic cytotoxicity and the latter almost entirely antagonistic cytotoxic effects in hGES.

173. Bensassi *et al.*, (2014) investigated the cytotoxic effect of DON and ZEN, alone or in combination in human colon carcinoma cells (HCT116) using the fluorescent probe fluorescein diacetate assay. Cells were treated with 100 µM DON or 40 µM ZEN or DON and ZEN in combination at 100 µm and 40 µm, respectively for 48 hours. The co-exposed mixture was observed to have an antagonistic effect (~20% mortality), since groups exposed to either DON or ZEN alone caused a greater cytotoxic effect at ~35 and 30%, respectively.

174. Alassane-Kpembi *et al.*, (2013) assessed the interactions caused by co-exposure to Type B trichothecenes, alone or in binary mixtures on proliferating Caco-2 cells. The MTT and neutral red assays were used to measure cytotoxicity. Cells were exposed to DON and NIV at different concentrations with a 1:0.8 ratio (DON: 0, 0.25, 0.5, 1.0, 2.0, 4.0 µM and NIV: 0, 0.2, 0.4, 0.8, 2.6, 3.2 µM) for 48 hours. The authors observed a cytotoxic synergistic effect at low concentrations of DON (up to 0.5 µM) and an additive effect at higher concentrations. In the case of DON + 15-ADON, the authors treated the cells with mixtures of the mycotoxins at the same concentrations (0.375, 0.75, 1.5, 3, 6 ppm). A synergistic effect was also observed for this combination.

175. Ficheux *et al*., (2012) studied the myelotoxicity of different combination of *Fusarium* mycotoxins (DON + ZEN, T-2 + ZEN, DON + T-2 and DON + FB_1) using *in vitro* human granulo-monocytic (Colony Forming Unit-Granulocyte and Macrophage; hCFU-GM) haematopoietic progenitors. The haematopoietic progenitors were treated with mixtures of mycotoxins at different concentrations for 14 days (*Table 4*). The observed results for that majority of binary combinations were mainly additive/synergistic apart from DON and FB_1 which caused antagonistic effects.

Table 4 - Presents the binary combination effects of fusarium mycotoxins: deoxynivalenol and zearalenone; T-2 toxin and zearalenone; deoxynivalenol and T-2 toxin and; deoxynivalenol and fumonisin B1 in human granulo-monocytic haematopoietic progenitor cells (adapted from Ficheux *et al*., 2012).

Mycotoxin mixtures	Cell model	Mycotoxin concentration	Combination effects
DON +ZEN	hCFU-GM	DON (40-100 nM) + ZEN (0.2 µM) or DON (40 nM) + ZEN (1-10 µM)	Additive
T-2 + ZEN		T-2 (0.5-1.6 nM) + ZEN (0.2 µM) or T-2 (0.5 nM) + ZEN (1-10 µM)	Additive
DON + T-2		DON (40-100 nM) + T-2 (0.5 µM) or DON (40 nM) + T-2 (1-1.6 µM)	Additive or synergistic
DON + FB_1		DON (40-100 nM) + FB_1 (0.5 µM) or DON (40 nM) + FB_1 (1-2 µM)	Antagonistic

Abbreviations: DON= Deoxynivalenol; Fus-X= Fusarenon-X; NIV = Nivalenol; hCFU-GM = Human colony forming unit-granulocyte and macrophage cells.

176. Kouadio *et al*., (2007) further studied the cytotoxicity, lipid peroxidation, DNA synthesis, and DNA fragmentation of DON and FB_1 in human Caco-2 cells. Cells were exposed to different concentrations of DON at 4, 10, 20 µM and 10 µM FB1 (alone or in combination) for 72 hours in cytotoxicity tests. Whilst for DNA synthesis, DNA fragmentation and lipid peroxidation, DON and FB_1 were added to cells at 10 µM each (alone or in combination) for 24 hours. The results determined a synergistic effect for cytotoxicity, less than additive for DNA synthesis, additive for DNA fragmentation and synergistic for lipid peroxidation.

In vivo

177. Bracarense *et al*., (2012) investigated the effects of DON and FB, alone or in combination on some intestinal parameters including morphology, histology and expression of cytokines and junction proteins.

178. Male crossbred castrated piglets (n=24) were randomly assigned to four different groups, receiving separate diet for 5 weeks: control diet (0.5 mg DON/kg feed, FB below the limit of detection); diet containing 2.8 mg DON/kg feed; diet containing 5.9 mg FB/kg feed (4.1 mg FB_1 + 1.8 mg FB_2); diet containing 3. 1 mg DON and 6·5mg FB/kg feed (4.5 mg FB1 + 2.0 mg FB_2). These diets were artificially contaminated with the fungal culture. It was estimated that piglets were exposed to 130 and 260 µg/kg bw/day of DON and FB, respectively. DON, ZEN and enniatin were found to be naturally present in the cereals used, resulting in the concentrations of 500, 50 and 100 µg/kg feed, respectively.

179. The authors observed varying effects that were dependent on the parameters studied and segments of intestine assessed: synergistic in number of goblet cells and eosinophils in the ileum (immune cells); additive in the expression of IL-10, TNF-α and adherent proteins (cytokines and junction protein expression); less than additive in expression of IFN-γ and in arbitrary lesion scores (histological lesions and cytokine expression); antagonistic for some cell populations (goblet cells, plasma cells, eosinophils and lymphocytes in the jejunum) and some cytokine expression (IL-1β and IL-6).

Summary of Fusarium and their interactions with each other

180. Various studies of *Fusarium* mycotoxins and their interactions with each other (FB_1, ZEN, T-2, DON, 15-AcDON and NIV) utilising different methodologies and assessment of different endpoints have been summarised above. It should be highlighted that most studies were carried out using *in vitro* test methods.

181. A summary table is presented in the next page to provide a highlight overview of the reviewed literature (*Table* 5).

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	Ficheux *et al*., (2012)
	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.

Other considerations

Potential adverse effects on the microbiota as a result of mycotoxin exposure

182. Liew & Mohf-Redzwan (2018) reviewed the role of mycotoxins (trichothecenes, ZEN, FBs, OTA, and AFs) toward gut health and gut microbiota. In general, the authors observed that a bi-directional relationship exists between mycotoxins and gut microbiota, which suggested that the gut microbiota might be involved in the development of mycotoxicosis. In this review, the combination of mycotoxins was explored. Two studies *in vivo* studies for female gilts[29] (Piotrowska *et al*., 2014) and in calves (Baines *et al*., 2013) were briefly discussed.

183. Female gilts were exposed to 40 µg ZEN/kg bw and 12 µg ZEN/kg bw (were described as no observable adverse effect level doses) for 42 days. A significant decrease in *Clostridium perfringens, Escherichia coli (E. coli)*, and other bacteria in the *Enterobacteriaceae* family was observed in the ascending colon after the experiment, however, the overall biodiversity of microorganisms and the metabolism of amino acids in the gut were increased. It was suggested that the increased metabolism of amino acids may exert adverse effects due to the formation of biogenic amines and other pro-carcinogenic compounds (Piotrowska, *et al*., 2014).

184. Baines *et al*., (2013) investigated the potential role of mycotoxins (AFB_1; 1-3 ppb and FB_1; 20-3250 ppb) in mediating infection of Shiga toxin-producing *E. coli* (STEC) in immature calves. The composition of STEC-secreted cytotoxin was affected as reflected in the elevated concentration of intracellular Ca2+ with a corresponding increase in cytotoxicity.

Potential endocrine effects of mycotoxin exposure

185. Demaegdt *et al*., (2016) assessed the endocrine actives of 13 mycotoxins and their mixtures on the oestrogen, androgen, thyroid and peroxisome proliferator-activated (PPARγ2) receptors using luciferase reporter cell lines.

186. The 13 mycotoxins were ZEN, α-ZEN, β-ZEN, DON, 3-AcDON, 15-AcDON, NIV, Fus-X, ALT, FB_1, FB_2, OTA and CIT. ZEN and its metabolites were observed to have the strongest oestrogenic potency (EC_{50} 8.7 10^{-10}, 3.1 10^{-11}, and 1.3 $^{10-8}$, respectively). 3-AcDON was also shown to have exhibited oestrogenic activity at EC50 3.8 10^{-7}. The potency order was: α-ZEN > ZEN >> β-ZEN > 3-AcDON ~ 15-AcDON > ALT.

[29] A gilt pig is a female under the age of 1 year. Generally, the term refers to a pig who has not farrowed or given birth to a litter.

187. The potency order of the antagonistic activity on the androgen receptor was: 15-AcDON > 3-AcDON ~ α-ZEN ~ DON ~ Fus-X ~ ZEN ~ ALT ~ OTA > β-ZEN > NIV.

188. The potency order of the antagonistic activity on the thyroid receptor was: DON > OTA > α-ZEN > 3-acetyl-DON > ZEN ~ b-ZEL.

189. The potency order for antagonism on the PPARγ2 was: 15-AcDON ~ DON > OTA ~ NIV > 3-AcDON ~ Fus-X ~ CIT > FB_2 ~ ZEN ~ α-ZEN.

190. Six mixtures were assessed: OTA + CIT; ZEN + α-ZEN + β-ZEN + DON + 3-AcDON + 15-AcDON; DON + 3-AcDON + 15-AcDON + NIV + Fus-X; DON + 3-AcDON + 15-AcDON; ZEN + α-ZEN + β-ZEN (which all used equimolar ratios) and ZEN + α-ZEN + β-ZEN at EC_{50} ratios. Most of the mixtures reacted as predicted by the concentration addition (CA) theory. The dose-response curves of the predicted CA were close to the observed ones (within the 95% confidence interval).

191. The authors concluded that mycotoxins do interact with oestrogen receptors, as well as other nuclear receptors *in vitro* and although these observations may not be directly extrapolated to observations *in vivo* it allows a basis for further research.

Summary of toxicology

192. The toxic effects of some binary mycotoxins were discussed above (*e.g.* AFB_1 and FB_1, OTA and DON *etc*). 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; this indicated a range of antagonistic to synergistic effects based on the observed endpoint. *In vivo* rat studies for hepatotoxicity of AFB_1 and FB_1 were observed to be synergistic in general.

193. *In vitro* studies suggested that combinations of AFB_1, OTA and DON with other mycotoxins are synergistic in general.

194. The available data suggest that the toxicity of combinations cannot be predicted based on the toxicity of individual mycotoxins. Furthermore, there is a large amount of variability in the methodology used since there is currently no harmonisation on combinative testing strategies for each toxicological endpoint. Combinations of mycotoxins may also have potential adverse effects on the microbiota and the endocrine system.

Exposure assessment

195. In the context of this COT paper, the aggregate dietary exposure to mycotoxins is defined as when an individual is exposed to one or more mycotoxins from one food source (*e.g.* a cereal bar) or multiple food sources (*e.g.* cereal bar and bread), thus leading to an aggregate dietary exposure.

196. 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. An aggregate dietary exposure is therefore highly likely in humans due to varied diets and/or one food commodity may be contaminated with more than one mycotoxin.

197. 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 in the Methods for sampling and measuring mixtures of mycotoxins in food matrices section, 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.

198. As such, the Secretariat has decided that a full assessment for the aggregate dietary exposure of mycotoxins should not be carried out until advice from the COT members has been sought. This section will outline a suggested stepwise approach to the exposure assessment. The approaches are based on use of deterministic and if necessary, probabilistic approaches.

Stepwise approach

199. A stepwise approach to the exposure assessment is proposed in the following paragraphs.

200. 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.

201. 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.

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

Potential data sources

203. Three data sources to use in the exposure assessment are proposed in the following paragraphs.

Food Standards Agency – Mycotoxin Total Diet Study

204. The FSA has previously carried out a Total Diet Study (TDS)[30] that included mycotoxin analysis in 2011, 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).

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

206. The following limitations were observed including; 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. Finally, as mentioned multi-mycotoxin analysis was not consistently used.

207. Further information received from the project manager; Susan 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

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

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 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.

European Food Safety Authority – MYCHIF platform

208. 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.

209. 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: component-based and provisional daily intake approaches.

210. In the component-based approach, 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).

211. 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[31] 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.

212. 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.

213. 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 literature (i.e. non-UK data)

214. 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 66 mycotoxicosis in humans as a result from co-exposure to co-contaminated food as reported in paragraph 96.

215. For the exposure assessment, non-UK co-occurrence data and 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.

216. 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.

[31] 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.

Probabilistic modelling using either a component-based or provisional daily intake approach

217. As mentioned previously, an approach to probabilistic modelling is further considered and is proposed in the next paragraphs.

218. As seen in the MYCHIF report (see paragraph 209 onwards), modelling was carried out using both component-based and provisional daily intake approaches (Battilani *et al*., 2020).

219. The most studied mycotoxin biomarker in humans is AFs, followed by 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.

220. Even though biomarker studies on mycotoxins have been increasing, 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.

221. Kifer *et al*., (2020) published a review that discussed the advantages and disadvantages of available mathematical models on assessing the effect of mycotoxin combinations. Based on their review of the published *in vitro* combined mycotoxin studies (n=35), the most commonly used model in mycotoxicology are factorial analysis of variance and additivity (*i.e.* simple addition of observed effects). They concluded these methods have been incorrectly based on the assumption that mycotoxin dose-effect curves are linear – and so, many conclusions on the reported interaction (*i.e.* describing synergism, antagonism or additive effects) may have been derived incorrectly.

222. The authors noted that the only appropriate approach to assess the nature of an interaction is to correctly estimate the dose-effect curves of each mycotoxin and combination, and apply a well-defined model (based on either Bliss[32] or Loewe's theory[33]) with respecting the model's assumptions and fitting the model by a direct estimation of all model parameters from a non-linear least squares fitting. It was

[32] Bliss Independence Criterion is a model that predicts the proportion of animals that will die after combining two toxins under the assumption that there is no interaction between the toxins – implying that they have completely different mechanisms of action or act in different compartments.
[33] Loewe's Additivity Law (also called iconographic method, concentration additivity or dose additivity) assumes that mycotoxins act within the same compartment by the same mechanism. The only difference between the mycotoxins is their potency.

further suggested that, results should be presented in a simple and clearly defined way (*e.g.* an isobologram or combination index).

Summary of exposure assessment

223. 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. A stepwise approach to an exposure assessment was presented as well as a description of three potential data sources that could be utilised within this approach. These were the FSA – TDS Mycotoxin report, the EFSA MYCHIF platform and the utilisation of available co-occurrence data reported within the literature (*i.e.* non-UK relevant data). The possibility of probabilistic modelling using either a component-based or provisional daily intake approach was also proposed to be used additionally if required.

Risk assessment

224. It is generally accepted that the effects of a combination of mycotoxins cannot be predicted based solely on their individual effects and that, in addition to additivities and synergies, there can also be antagonisms observed.

225. 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.

226. 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.

227. Assunçao *et al*., (2016) have described the following issues and/or data gaps concerning the risk assessment of multiple mycotoxins (*Figure 2*). The summary is provided in sections: hazard, exposure and risk assessment, and conclusions.

228. The main challenges in hazard assessment of multiple mycotoxins include; the lack of accurate information regarding the bioaccessibility of mycotoxin mixtures (*i.e.* the actual percentage of mycotoxins that can be absorbed in the small intestine) that would enable a more accurate 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 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.

229. It was suggested by Assuncao *et al*., (2016) that priorities for hazard assessment needs to be set in order to put more resources and efforts into the most relevant mixtures. Rationales for prioritisation include; the frequency of co-occurrence in food (and feed), the hazardous potency of the single toxins, the structure-activity relationship pointing to a strong probability of interactions or on preliminary data suggesting synergistic effects. Even though the available data on the combined toxic effects is increasing, a considerable degree of inconsistency is noted when comparing outcomes for studies focusing on similar mixtures.

230. In a general framework, the main difficulty of performing a dietary exposure assessment is the handling of concentration data that is below the limit of detection (LOD) of the analytical method. These data are commonly substituted with upper and lower bound values. It was highlighted that, a representative food sampling design (selection of most susceptible foods/composite samples), an accurate chemical analysis method (with low detection limits) and a suitable method to manage left-censored data are the key decisive steps for obtaining a realistic exposure estimates (with a low level of uncertainty).

231. The authors then proceeded to discuss approaches for carrying out an exposure assessment and their limitations. It was suggested that probabilistic modelling should be carried out when refinements are required, particularly for high quartiles of the exposure group. Should assessors carry out an indirect approach obtained with the combination of data of mycotoxin occurrence in food and food consumption patterns the following limitations must be kept in mind: the heterogenous distribution of mycotoxins in food, the possible routes of exposure (*i.e.* does not consider potential inhalation or dermal exposure), the presence of masked mycotoxins, the influence of food processing, inter-individual variation in ADME parameters, and the under- and overestimation in food consumption data. Overall, these limitations could lead to an under-and/or overestimation of the exposure.

232. As such, the use of biomarkers has been proposed as a suitable alternative for establishing the real human exposure to mycotoxins. Although this approach also has its challenges and limitations. It has already been mentioned that there is a lack of toxicokinetic data on mycotoxins in humans. The following uncertainties were also highlighted to keep in mind when performing a risk assessment based on urinary

mycotoxin levels; difficulties to correlate human dietary habits, concurrent mycotoxin contamination of food, and consequent presence of these mycotoxins in human urine. Further uncertainties relating to data comparison between different studies include; the differences in age groups, detection limits, the number of subjects included in the study, and the analytical performances of the used methods.

233. In terms of risk assessment, one of the main challenges posed to risk characterisation is the absence of toxicological data. The few works that published the application of similar methodologies in order to evaluation the risk to simultaneous exposure to different mycotoxins were observed to utilise different terminologies. It was therefore suggested that the use of harmonised terminology, approach and methodology would improve datasets that would allow for a more robust risk assessment.

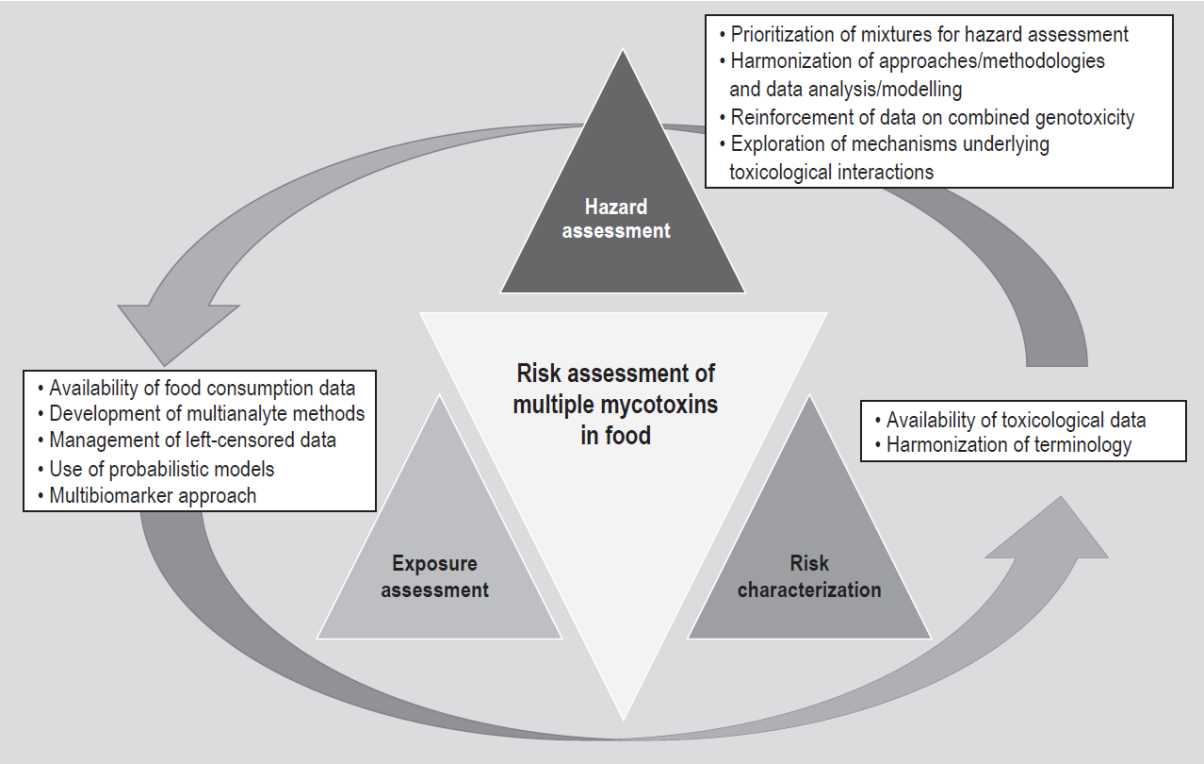

Figure 2 presents a circular flow diagram that describes a holistic overview on the inter-relationship between different steps of multiple mycotoxins health risk assessment and their respective challenges (reproduced from Assunçao *et al*., 2016).

234. To conclude, the authors recommended that further studies on hazard and exposure assessment of multiple mycotoxins, using harmonised methodologies, are crucial towards an improvement of data quality and a more reliable robust risk characterisation. Additionally, a deeper understanding of the interactions between multiple mycotoxins will assist in drawing real life conclusions on the health impact of human exposure to mycotoxin mixtures.

235. 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 carried out presently.

Further considerations

236. In terms of the potential effects of climate change on fungi and host specific interactions; 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. As a result, new combinations of mycotoxins/host plants/geographical areas may arise. It was recommended that developments of new diagnostic tools, a deeper knowledge of both biology, and genetics of toxigenic fungi may be required.

237. 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) (pre-print) whom performed a longitudinal assessment of mycotoxin co-exposures in exclusively breastfed infants in Austria.

Summary and conclusions

238. 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.*

239. 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 where 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.

240. An external EFSA report by Battilani *et al*., (2020) was recently 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.

241. 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.

242. For the component-based approach 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.

243. In terms of data gaps, there is still a limited knowledge on the presence and co-occurrence of multiple mycotoxins, both for native 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.

244. 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 FB_1 and AFB_1 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).

245. The main analytical methods to measure mycotoxins 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.

246. 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.

247. In terms of toxicokinetic data, only on 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.

248. The toxic effects of some binary mycotoxins were discussed in this paper (*e.g.* AFB_1 and FB_1, OTA and DON *etc*). 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.

249. It seems that the toxicity of combinations cannot be predicted based on the toxicity of individual mycotoxins. Furthermore, there is a large amount of variability between each methodology carried out for studies since there is currently no harmonisation on combinative testing strategies for each toxicological endpoint.

250. In terms of exposure assessments, the use of biomarkers data was explored in the MYCHIF report (see paragraphs 39-41 and 209-213). 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, the excretion rate, and a consensus of a validated biomarker to be used in context to a multi-mycotoxin analysis.

251. A stepwise approach for the exposure assessment was detailed (see paragraphs 199-202) 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), exposure data derived from literature.

252. A risk assessment could not be carried out on the potential risks to aggregate exposure of mycotoxins for several reasons (*Figure 2*). 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. 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.

253. 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.

Questions on which the views of the Committee are sought:

254. Members are invited to consider the following questions:

i). From the information above, do the Committee agree that available data are not sufficient to perform a risk assessment?

ii). If yes, which data gaps(s) do the Committee consider to be more important, so that a risk assessment may be performed?

iii). What future research would the Committee recommend?

iv). What are the Committees views on the proposal for exposure assessment? Is the quality of the proposed databases sufficient and applicable for our purposes?

v). Does the Committee have any other comments?

Secretariat
July 2020

Abbreviations

2-AAF	2-acetylaminofluorene
3-AcDON	3-acetyldeoxynivalenol
4,15-DAS	4,15-diacetoxyscirpenol
15- AcDON	15-acetyldeoxynivalenol
AFs	Aflatoxins
ALT	Alternaria
CA	Concentration Addition
CIT	Citrinin
COT	Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
DAS	Diacetoxyscirpenol
DEN	Diethylnitrosamine
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
EC	European Commission
EDL	Effective Dosage Level
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
ENNs	Enniatins
EU	European Union
FAO	Food and Agriculture Organization
FB1	Fumonisin B_1
FBs	Fumonisins
FSA	Food Standards Agency
Fus-X	Fusarenon-X
GC	Gas Chromatography
GST-P+	Glutathione S-transferase
hBEAS-2B	Human Bronchial Epithelial Cells
hHep-G2	Human Hepatoma Cells
hLF	Human Lung Fibroblasts
hGES-1	Human Gastric Epithelial Cells
HPLC	High Performance Liquid Chromatography
Hsp70	Heat Shock Protein 70
hUVEC	Human Umbilical Vein Epithelial Cell
IA	Independent Addition
IAC	Immunoaffinity Columns
IC_{50}	Inhibitory Concentration, 50%
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS	Liquid Chromatography-Mass Spectrometry
LD_{50}	Lethal dose, 50%
LOD	Limit of Detection

LOQ	Limit of Quantification
MFC	Multifunctional Columns
MOE	Margin of Exposure
MS	Mass Spectroscopy
MTT	Methyl Thiazol Tetrazolium
MYCHIF	Mycotoxin Mixtures in Food and Feed: Holistic, Innovative, Flexible Risk Assessment Modelling Approach
MYTOX	Mycotoxin and Toxigenic Research Group
NEO	Neosolaniol
NIV	Nivalenol
OTA	Ochratoxin A
PAT	Patulin
PK	Porcine Kidney cells
PPARγ2	Peroxisome Proliferator-Activated Receptor
ROS	Reactive Oxygen Species
SCF	Scientific Committee on Food
TDS	Total Diet Study
TEA	Tenuazonic Acid
UK	United Kingdom
WHO	World Health Organization
ZEN	Zearalenone

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