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Human and animal health risk assessment of mycotoxin mixtures in maize: from fungal production and occurrence to harmonised risk characterisation

Coordinator: Ch.mo Prof. Marco Trevisan

Candidate: Roberta Palumbo
Matriculation n.: 4612269

Tutor: Prof.ssa Paola Battilani
Co-tutor: Dr. Jean-Lou Dorne

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Table of contents

General introduction

Mycotoxins are naturally-occurring toxins produced by fungi. They are undesirable contaminants widely occurring in feed and food commodities, causing adverse effects in animals and humans. Mycotoxins are produced mainly by fungal species belonging to three genera: *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp., and on a minor extend by *Alternaria* spp. and *Claviceps* spp. Mycotoxigenic fungi are commonly not host specific, but they are found more often on a particular crop if the ecological conditions in the cropping area are suitable for their growth. Different fungal species may simultaneously infect the same crops in the field, or different mycotoxins can be synthesised by the same fungi on the crop, leading to the co-occurrence of multiple parental mycotoxins. In addition, many structurally-related congeners defined as modified mycotoxins are generated by plant and fungi metabolism, or food processing, and coexist with their native forms (Rychlik et al., 2014). As a consequence of their ubiquitous presence, co-contamination of agricultural products with multiple mycotoxins is frequently observed and recently stressed.

Mycotoxins are well established to have a number of health impacts both in humans and animals. The exposure risk to human is directly through foods of plant origin (i.e. mainly cereal grains) or indirectly through foods of animal origin (i.e. milk, eggs, etc.). Depending on the quantities consumed, mycotoxins and their metabolites are associated with severe acute poisoning, including death, and chronic adverse health effects. In addition, interaction effects (i.e. additive, synergistic, or antagonistic) have also been associated with the co-exposure to multi-mycotoxin.

The control of risks possibly associated with mycotoxin contamination in food and feed is a priority of the European Union (EU). In fact, European legislation protects consumers by setting legal maximum levels (MLs) for main classes of mycotoxins in food and feed to ensure they are not harmful to human or animal health (European Commission, 2006b, a, 2010, 2011, 2013). However, the current maximum permitted levels of mycotoxins in EU legislation do not consider the sum of mycotoxins produced by different fungi species and they are either based on the risk assessment of a single compounds or on their sum like in the cases of aflatoxins (AFs) and fumonisins (FBs), where respectively the sum of AFB_1, B_2, G_1, G_2 and FB_1, FB_2 is applied (European Commission, 2006b). Guidance values for FB_1+FB_2 have been recommended in products intended for animal feed in the EU (European Commission, 2016). This approach underestimates the total amount of mycotoxins that humans and animals are exposed to and ignores the

possibility of interacting effects of mycotoxins in the organism underestimating the final adverse effect(s).

It is increasingly realized that not only the formation and co-occurrence of parent compounds but also of their modified forms must be regarded as a relevant contribution to the overall toxic load (EFSA, 2016b, a, 2017c, d, a, 2018). Thus, the impact of mycotoxin mixtures on animal and human health has been recognised by European regulatory bodies as an emerging risk for feed and food safety and security. In this respect, efforts have continued internationally during the last years to keep mycotoxin levels as low as reasonably achievable following recommended good practices both at pre-harvest (i.e. good agricultural practices - GAPs), and post-harvest (i.e. storage and processing practices). However, due to the difficulty in depicting the biosynthesis of mycotoxin mixtures and their realistic co-occurrence, as well as the lack in toxicological data, implementation remained vague and methods for carrying out risk assessment for combined exposure to multiple mycotoxins have not been adequately implemented until now.

Over the last decades, the European Food Safety Authority (EFSA) has been very active in the area of human and animal risk assessment of mycotoxins (Eskola et al., 2018), producing scientific opinions dealing with well characterized mycotoxins (e.g. AF, deoxynivalenol (DON), T-2 and HT-2 toxins, zearalenone (ZEN), FBs, etc.) (EFSA, 2004b, a, c, 2005, 2006, 2007, 2011, 2013c, a, 2017b, c, d, 2018) and emerging mycotoxins (e.g. alternaria toxins, beauvericin (BEA), Enniatins (ENNs), etc.) (EFSA, 2014b). The development, validation, implementation and harmonisation of methodologies and approaches for the assessment of health risks for humans, animals, plants and the environment is one of the key strategic objectives of EFSA, being the assessment of chemical mixtures an area which EFSA considers as a challenge for the future. In this respect, EFSA has initiated a series of projects dealing with the development of harmonised methodologies for combined exposure to multiple chemicals that have led to the formulation of several recommendations to further develop methods for the risk assessment of mixtures (Battilani et al., 2012; EFSA, 2013b, 2014a). These include the refinement of: (i) detection and reporting of occurrence data in food and feed commodities to determine realistic mixtures of mycotoxins; (ii) scientific basis to set assessment groups (AGs) for chemicals based on their elimination patterns in a number of organisms (toxicokinetics - TK) and their combined toxicity profiles (dose addition, response addition, or interaction) to identify unknown modes of actions for further refinement of hazard characterization; (iii) combining the

refinements of (i) and (ii) for risk characterisation and uncertainty analysis based on realistic mixtures in food and feed (EFSA, 2013b, 2014a).

In 2019, EFSA published a guidance document on harmonized framework for risk assessment of combined exposure to multiple chemicals for human and animal health (EFSA, 2019). This framework consists of well-defined steps of risk assessment meaning that once the issue to be addressed is identified (problem formulation), exposure is determined (exposure assessment) and toxicological effects identified (hazard identification and characterization), both information are compared so that the risk to human and animal health can be characterised (risk characterization). This approach has been applied to a number of case studies dealing with several chemicals, mainly pesticides or food additives (EFSA, 2008a; WHO, 2009; EFSA, 2013c, b). Finally, an EFSA funded project started in 2017 on developing a holistic innovative and flexible risk assessment modelling approach for mycotoxin mixtures in food and feed, and it has been recently concluded (i.e. MYCHIF project).

The present thesis has been conducted within the MYCHIF project, and it aims to apply a holistic approach for the risk assessment of mycotoxin mixtures in food and feed, i.e. from fungal production and occurrence to harmonised risk characterisation. This was done in three folds. Firstly, available environmental, ecological, and agronomic factors that may affect the relative abundance of co-occurring mycotoxins in the contaminated crops were collected from peer-reviewed literature, with focus on maize (Chapter I). Secondly, (co-)occurrence data on mycotoxins in core cereals was extracted from available articles in the scientific literature and analysed to estimate potential pattern of co-exposure in humans and animals (Chapter II). Finally, Chapter III investigates the applicability of the EFSA guidance to multiple mycotoxins through a scenario of possible co-exposure in humans and animals, using maize as a case study. In particular, a human and animal risk assessment to mycotoxin mixture in maize was conducted using a modelled component-based approach for selected mixture of mycotoxins, that, according to our data, co-occur in maize based feed and food products. Figure 1 summarises the outline of the thesis.

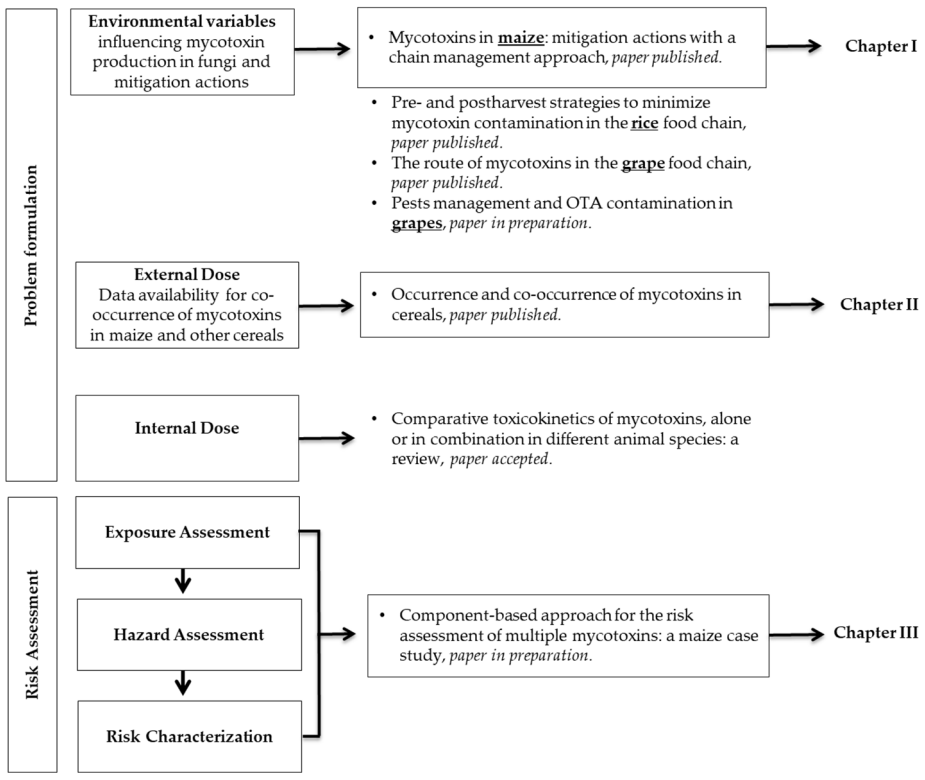

Figure 1: Overview of the outline of the thesis. The flow chart shows the ranking of the chapters in relation to the step-wise approach proposed by EFSA (EFSA, 2019).

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Chapter I - Mycotoxins in maize: mitigation actions with a chain management approach

Roberta Palumbo [1], Ana Gonçalves [2], Athanasios Gkrillas [3], Antonio Logrieco [4], Jean-Lou Dorne [5], Chiara Dall'Asta [3], Armando Venâncio [2] and Paola Battilani [1]

[1]Faculty of Agriculture, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29100 Piacenza, Italy
[2]CEB - Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal
[3]Università degli studi di Parma, Via Università 12, 43121, Parma, Italy; chiara.dallasta@unipr.it
[4]National Research Council, Institute of Sciences of Food Production, via Amendola 122/O, 70126, Bari, Italy
[5]European Food Safety Authority (EFSA), Via Carlo Magno 1A, 43126, Parma, Italy

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SUMMARY

Maize is the principal staple food/feed crop exposed to mycotoxins, and the co-occurrence of multiple mycotoxins and their metabolites has been well documented. This review presents the infection cycle, ecology, and plant-pathogen interactions of *Aspergillus* and *Fusarium* species in maize, and current knowledge on maize chain management to mitigate the occurrence of aflatoxins and fumonisins. Preventive actions include at pre-harvest, as part of cropping systems, at harvest, and at post-harvest, through storage, processing, and detoxification to minimize consumer exposure. Preventive actions in the field have been recognized as efficient for reducing the entrance of mycotoxins into production chains. Biological control of *Aspergillus flavus* has been recognized to minimize contamination with aflatoxins. Post-harvest maize grain management is also crucial to complete preventive actions, and has been made mandatory in government food and feed legislation.

Keywords: *Aspergillus*; *Fusarium*; aflatoxins; fumonisins; deoxynivalenol.

INTRODUCTION

Maize is one of the most important cereals produced for human and animal consumption in the European Union (EU), and is grown mainly for grain and forage. More than 80% of maize grain is used for feed, and the rest is used for production of starch and semolina (Eurostat, 2019). In 2017/2018, the EU maize yields reached approx. 65 million tons (European Commission, 2019), approx. 5% of the global maize production. Maize is second to wheat in total EU cereal production (Statista, 2018). Since 2017, the EU has been importing significant volumes of maize, mainly coming from Ukraine, Brazil, and Canada. This is partly due to the increased demand for maize feed (+8%), and significant reductions in the production of barley and other cereals for feed consumption (European Commission, 2019). As well, there has been significant reduction in maize growing areas in some European countries, where mycotoxin contamination is a major concern. That is because of the economic losses caused by discarded lots that are non-compliant with legal mycotoxin limits, and the consequent income uncertainty for farmers.

Maize is exposed to mycotoxins, which are secondary metabolites of fungi with toxic effects on humans and animals, and which cause illnesses and also economic losses. Mycotoxin contamination is the major non-tariff trade barrier for agricultural products, which negatively impacts the health and income of small-holder farmers, regional and international trade, and the world economy (Logrieco *et al.*, 2018). A range of toxic effects has been associated with exposure to mycotoxins in humans and in many animal species (Eskola *et al.*, 2018). Hence, the maximum concentrations of the main class of mycotoxins in agricultural food and feed products, as well as in their commodities, are regulated in Europe, or recommendations are listed for animal consumption (Commission Regulation (EU) 576/2006; Commission Regulation (EU) 1881/2006; Commission Regulation (EU) 574/2011; Commission Recommendations (EU) 165/2013).

One of the major issues in the contamination of maize is infection with *Aspergillus flavus* and *Aspergillus parasiticus,* and the resulting occurrence of aflatoxins (AFs). In addition, the occurrence of aflatoxin B1 (AFB1) in feed can lead to contaminated milk, because the toxin is metabolized to aflatoxin M1 (AFM1) by dairy cattle when fed with contaminated feed, and there is carry-over to dairy products (EFSA, 2004; van der Fels-Klerx and Camenzuli, 2016).

Fusarium species also infect maize and contaminate grains with mycotoxins, which include deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FBs), nivalenol (NIV), T-2 toxin (T2), and HT-2 toxin (HT2). In maize the co-occurrence

of AFs and FBs is common (Camardo Leggieri *et al.*, 2015). Although there are no data demonstrating significant interaction between these toxins, reports suggest that both additive and synergistic interactions may occur (Torres *et al.*, 2015; Abbès et al., 2016; Qian et al., 2016). Mycotoxins are very stable compounds and accumulate in maize grain in the field after fungal infections during the crop growing season, with possible post-harvest increases when the environment remains suitable for fungal activity. Main factors affecting maize infection are: environmental conditions, plant susceptibility (depending on crop genetics and health status) as well as insect populations.

Many efforts have been devoted to develop strategies, both at the pre- and post-harvest crop stages, to reduce production and occurrence of these mycotoxins in maize, and their entry into the food and feed chains. The present provides an account of advances since 2000 in strategies to reduce the occurrence of AFs, FBs, and DON across the maize supply chain.

ASPERGILLUS AND *FUSARIUM* SPECIES IN MAIZE

Many of the most relevant mycotoxins in maize are synthesized by two fungal genera: *Aspergillus* and *Fusarium*. *Aspergillus* spp. include all validated AF-producing fungi and most of the known species belong to the *Aspergillus* section *Flavi*, including *A. flavus* and its close relative *A. parasiticus*. *Aspergillus flavus* and *A. parasiticus* are very similar species of the section, sharing 96% DNA similarity of the aflatoxin gene clusters (Cary and Ehrlich, 2006). These species can be distinguished from one another using morphological and physiological characteristics, but *A. flavus* commonly only produces B series AFs, while *A. parasiticus* can produce both B and G series AFs. Non-aflatoxigenic strains also naturally occur in both species (Smith and Moss, 1985). *Aspergillus flavus* almost exclusively occurs in maize (Giorni *et al.*, 2007).

The most frequently isolated *Fusarium* species from maize are *F. verticillioides*, *F. proliferatum*, *F. graminearum*, and *F. subglutinans* (Leslie and Logrieco, 2014). These cause two different types of ear rot: (i) Fusarium ear rot or pink ear rot is caused primarily by members of the *Liseola* section, including *F. verticillioides*, *F. proliferatum* and *F. subglutinans*, now preferably referred to as the *Gibberella fujikuroi* species complex (GFsc); and (ii) *Gibberella* ear rot or red ear rot which is caused by species of the *Discolor* section, with *F. graminearum* being the prevalent species. *Fusarium verticillioides* and *F. proliferatum* can synthesize large amounts of FBs. Other species can be involved in the pathogenesis of maize ear rot, including *F. culmorum* and *F. equiseti* (Logrieco *et al.*, 2002). These two fungi produce trichothecenes (DON and NIV) and ZEN. Studies reporting the presence of *F.*

sporotrichioides and *F. langhsethiae* in maize are scarce (Görtz *et al.,* 2008), but these two species have been shown to produce T2 and HT2, and their roles in maize contamination with these two mycotoxins needs to be clarified. Recently, a new mycotoxin-producing species of *Fusarium*, *F. temperatum*, has been reported in Europe and South America by different authors. This species is morphologically similar and phylogenetically close to *F. subglutinans*, and has been reported as a producer of FBs, beauvericin (BEA), fusaproliferin (FUS) and moniliformin (MON) (Scauflaire *et al.,*2012; Fumero *et al.,* 2016).

Infection cycle of Aspergillus *and* Fusarium *species on maize*

Maize is susceptible to mycotoxin-producing fungi from flowering, at growth stage BBCH63 (male: beginning of pollen-shedding; female: when tips of stigmata are visible), and fungus infection efficacy is optimized at BBCH67 (female: stigmata drying) (Battilani *et al.,* 2003; Battilani *et al.,* 2013). *Aspergillus* and *Fusarium* species commonly reproduce by asexual spores (Battilani *et al.,* 2013). The conidia of *Aspergillus* are dispersed mainly by air movement (Battilani *et al.,* 2003). *Fusarium* species produce macroconidia which, for *F. graminearum,* are typically dispersed by splashing rain, and for the GFsc, also by air movement (Shaner, 2003; Paul *et al.,* 2004; Manstretta and Rossi, 2015; Manstretta and Rossi, 2016). Conidia in crop debris are considered the main sources of infection, and they enter host plants through natural openings or wounds (Cotten and Munkvold, 1998). Sexual reproduction is possible for Fusaria, and the relevance of this depends on the species and the crop location, while for *A. flavus* sexual reproduction has been demonstrated in the laboratory, and some evidence suggests that it could occur in nature although not yet observed (Horn *et al.,* 2009; Horn *et al.,* 2016).

Systemic development of *Fusarium* species from maize seeds and roots to the stalks and to cobs can also contribute to kernel infection, but the role of systemic infections remains to be confirmed (Munkvold *et al.,*1997; Murillo-Williams and Munkvold, 2008). Systemic infection by *Aspergillus* has never been considered.

Beside silk and systemic infection, insect-assisted infections by mycotoxigenic fungi have also been identified as important pathway for maize ear infections by *Aspergillus* and *Fusarium species*. Insects can be vectors of inoculum and host entry can be assisted by larvae feeding on kernels (Munkvold and Carlton, 1997). *Lepidoptera* typically have the greatest impacts on mycotoxin-producing fungi in maize. Much attention has been given to the interactions between *Lepidoptera*, including the European corn borer (ECB; *Ostrinia nubilalis*), and *F. verticillioides*

infections (Blandino *et al.,* 2015; Drakulic *et al.,* 2017). ECB is the main maize pest in Central and Southern Europe, and this insect has been shown to promote *F. verticillioides* and *F. proliferatum* infections in maize grains and consequent FB contamination, in temperate areas (Blandino *et al.,* 2015). The incidence of the western flower thrips (*Frankliniella occidentalis*) on maize ears has also been correlated with the presence of *F. verticillioides* (Parsons and Munkvold, 2012). Further evidence also indicates that kernel injury attributed to the western bean cutworm (WBC; *Striocosta albicosta*) can lead to increased levels of *F. verticillioides* and subsequent increased levels of FBs in maize (Parker *et al.,* 2017).

Ecology

Every fungal species has unique ecological requirements, and optimum conditions for fungal growth are not always those that are most appropriate to mycotoxin biosynthesis (Figures 1 and 2). Therefore, it is difficult to identify common ecological trends across different fungal species. Nevertheless, *A. flavus* is well adapted to warm and dry weather conditions (Giorni *et al.,* 2016). In contrast, the optimum conditions for the development of *F. verticillioides* include warm temperature (T) and moderate rainfall. Mild T and high rainfall during maize grain maturation are best for infections by *F. graminearum* (Bhatnagar *et al.,*2014). T, relative humidity (RH), and, above all, grain water activity (a_w) are the most important ecological factors influencing fungal colonization of maize grain substrates (Giorni *et al.,* 2011; Lazzaro *et al.,* 2012; Battilani *et al.,* 2016).
In vitro trials have indicated that the optimum a_w for growth of *A. flavus* is in the range of 0.96 to 0.98 at 25°C, 0.98 at 30°C, and 0.96 at 37°C (Pitt and Miscamble, 1995). In the field, *A. flavus* can grow in maize grain at a_w as low as 0.73 (8-12 % moisture content), and produce AFs down at a_w = 0.85 (17-19% moisture) (Giorni *et al.,* 2011; Battilani *et al.,* 2013; Battilani *et al.,* 2016). *In vivo* trials also shown that AFB1 is positively correlated with a_w when $a_w \geq 0.95$, confirming the *in vitro* data, and is negatively correlated when $a_w < 0.95$ (Giorni *et al.,* 2016). Therefore, a_w of 0.95 is proposed as a threshold, at which AF production increases rapidly. The influence of abiotic stresses on *A. flavus* infection is complicated by the co-existence of different fungal species in maize kernels during the crop growing season. Previous *in vitro* studies considered the competition between *F. verticillioides* and *A. flavus* (Giorni *et al.,* 2014). Dominance of one species over the other was demonstrated only under extreme conditions, while mutual antagonism was more common (Giorni *et al.,* 2016).
Growth of *F. verticillioides* occurs within a wide range of T, with an optimum T range of 22.5 to 27.5 °C and a minimum a_w = 0.87. The optimum T and a_w reported

for inducing FB production are from 20 to 25°C and 0.95 to 0.99 a_w, while no production was observed at 10°C and $a_w \leq 0.93$ (Medina *et al.,* 2013). *Fusarium temperatum* strains reached maximum growth rate at T values greater than 22°C and the least growth was at 15°C and 0.95 a_w, and these strains produced maximum amounts (1000 μg g^{-1}) of fumonisin B1 (FB1) at 0.98 a_w and 15°C (Fumero *et al.,* 2016). *Fusarium graminearum* grew over a wide range of T and moisture conditions, with the optimum growth at approx. 25°C and a_w = 0.977-0.995. The influence of incubation T (15, 20, 28, or 32°C) and a_w (0.96, 0.97, or 0.98) on the production on DON by *F. graminearum* on maize kernels was studied by Llorens *et al.* (2004). They demonstrated that a_w in the range considered did not significantly affect trichothecene synthesis, while T affected DON production with the optimum T being 28°C.

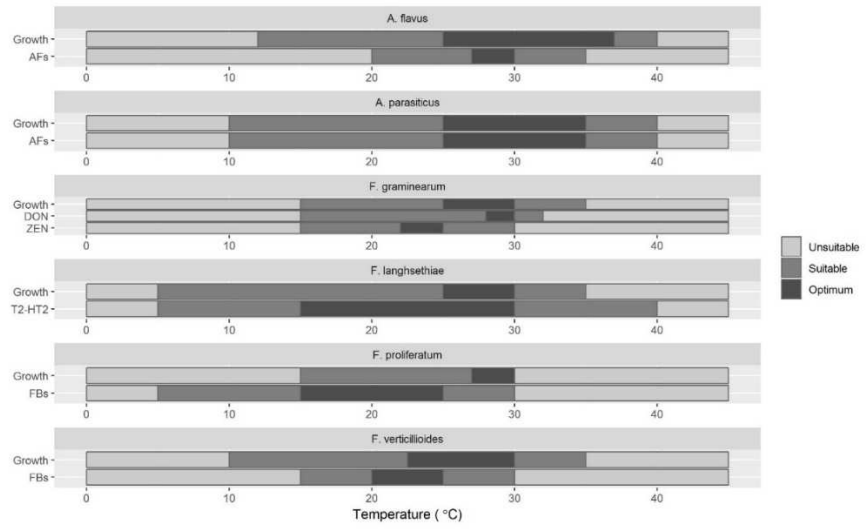

Figure 1: Temperatures (°C) required for fungal growth and mycotoxin production for *Aspergillus* and *Fusarium* species isolated from maize.

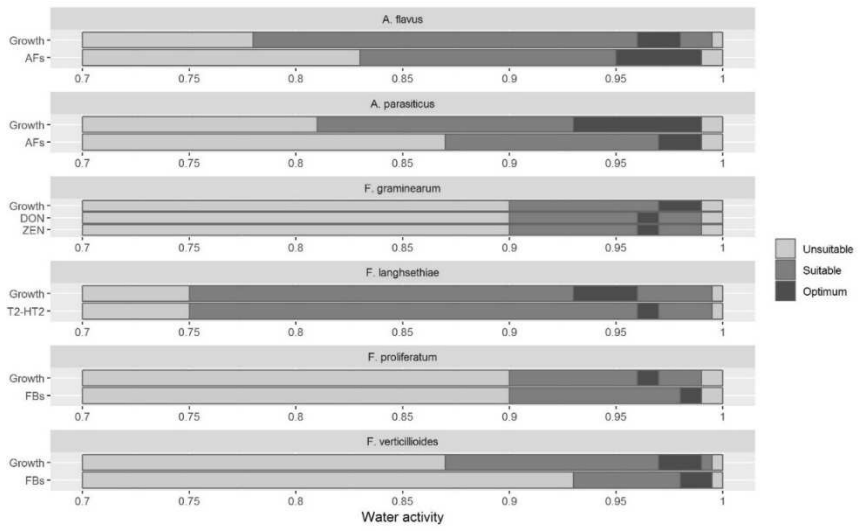

Figure 2: Water activity (a_w) required for fungal growth and mycotoxin production for same of the most relevant *Aspergillus* and *Fusarium* species isolated from maize.

Plant-pathogen interactions

Differences in chemical composition of maize kernels during each growing season and related plant physiology, can be variedly associated with fungal colonization and mycotoxin contamination (Luo *et al.,* 2008; Luo *et al.,* 2011).

The dynamics of a_w in grains during the growing season determines the competitiveness of *A. flavus* against other co-occurring ear rot fungi (Giorni *et al.,* 2011). The ability of *A. flavus* and other ear rot fungi such as *F. verticillioides* to utilize carbon sources at different T and a_w conditions could also influence the dynamics of AF contamination (Giorni *et al.,* 2016). Other factors, such as crop growth stage, physiology, active plant defenses, and grain composition, are also likely to influence the dynamics of AF production during grain ripening (Ojiambo *et al.,* 2018). The rate of drying of the ripening kernels critically affects their contamination with AFs and FBs (Medina *et al.,* 2013). The most significant increase in FB production and accumulation occurs after the dent stage. This stage is also characterized by acidification and maximum levels of amylopectin content; both of which enhance FB synthesis (Picot *et al.,* 2011).

Lipid composition of maize kernels also affects fungal infection and toxin accumulation by *Aspergillus* and *Fusarium* species (Dall'Asta *et al.,* 2012; Dall'Asta *et al.,* 2015; Battilani *et al.,* 2018). Plant and fungal oxylipins play crucial roles in

cross-talk between the pathogens and their host (Scala *et al.,* 2013; Ludovici *et al.,* 2014; Battilani *et al.,* 2018).

OCCURRENCE OF MULTIPLE MYCOTOXINS

A survey by Streit *et al.,* (2013) indicated that, on a global scale, 84% of maize was contaminated with at least one mycotoxin, and 46% was co-contaminated with multiple mycotoxins. The natural co-occurrence of mycotoxins produced by different fungi in maize and maize products has been reported, and most surveys have focused on the major mycotoxins AFs, FBs, ZEN, and trichothecenes (mainly DON) (Smith *et al.,* 2016; Ingenbleek *et al.,* 2019). Only a few studies have specified the percentage of the co-contaminated samples. Common co-occurrence of AFs + FBs, FBs + DON, and FBs + DON + ZEN has been reported (ranging from 25% to 40%). More details of the main reported mycotoxin combinations are summarized in Table 1.

Apart from the occurrence of parent forms, modified mycotoxins have been frequently reported to co-occur in cereals, including maize (Rasmussen *et al.,* 2012; Nakagawa *et al.,* 2013; Kovalsky *et al.,* 2016). Glucosides of DON, ZEN, and other minor trichothecenes have been frequently described. Mycotoxin modification in wheat is part of the biotransformation machinery expressed by host plants in response to pathogen attacks (Berthiller *et al.,* 2009a). However, toxin biotransformation has been little investigated in maize. Occurrence of modified FBs in maize has been reported (Bryła *et al.,* 2013a; Dall'Asta and Battilani, 2016), and conjugation of FBs with fatty acids (oleic and linoleic acids) through the formation of ester bonds has been described (Bartók *et al.,* 2010; Bartók *et al.,* 2013; Falavigna *et al.,* 2016). Recent evidence strongly supports the hypothesis that fatty acid esters of FB1 are produced by *F. verticillioides* using fatty acids from the substrate (Falavigna *et al.,* 2016). These compounds are formed by the fungus in a substrate concentration-dependent manner (Falavigna *et al.,* 2016), and they may undergo cleavage in the gastrointestinal tracts of mammals.

FBs can also occur as non-covalently bound forms, also known as "hidden fumonisins", now referred to as modified mycotoxins (Rychlik *et al.,* 2014). Several studies have demonstrated the complexation of FBs with maize macro-constituents, the main one being starch (Dall'Asta *et al.,* 2009; Dall'Asta *et al.,* 2010; Dall'Asta *et al.,* 2012; Bryła *et al.,* 2015). This complexity may significantly affect the quantification of FBs under routine conditions, requiring additional hydrolysis steps under alkaline conditions. The amounts of modified FBs are closely related

to environmental factors and chemical composition of maize, and may significantly contribute to the overall amount of FBs occurring in each sample. The ratio between free and total FBs has been reported at between 0.4 to 0.7, depending on yearly variations and host hybrid examined (Dall'Asta *et al.,* 2012; Bryła *et al.,* 2015; Giorni *et al.,* 2015). Dry milling of maize also increased free FBs in bran by 69% and total FBs partitioning in fractions by 46%, while free FBs decreased in flour by 28% and total FBs partitioning in fractions by 20% (Bryła *et al.,* 2015). Total release of this fraction under digestive conditions has been considered by the European Food Safety Authority. The contribution of modified FBs to overall FB exposure in animals, using an additional factor of 1.6 with respect to the free FB contents has been proposed. This factor has been extrapolated from several studies and a broad database (n = 316) (Dall'Asta *et al.,* 2010; Dall'Asta *et al.,* 2012; Bryła *et al.,* 2013b; Bryła *et al.,* 2014; Bryła *et al.,* 2015; Oliveira *et al.,* 2015).

In contrast to *Fusarium* mycotoxins, no modification of AFs in maize has yet been reported.

Table 1: Co-occurrence of mycotoxins in maize and derived products

Mycotoxin	Commodity	Observation	References
AFs; FBs	maize	- 95.6 % of samples with AFB1 and FBs (FB1+FB2)	Camardo Leggieri *et al.* (2015)
FBs; DON	maize products	- high co-occurrence of FB1, FB2 and DON - strong evidence of co-occurrence of FB1 and FB2	Cano-Sancho *et al.* (2012)
	maize and maize products	- 38 % of samples with FBs and DON	Kirincic *et al.* (2015)
	maize	- 25 % of samples with DON+FB1	Zachariasova *et al.* (2014)
FBs; BEA	maize	- 97 % of samples with FB1 and FB2 - 10 % of samples with OTA - 17 % of samples with BEA - 15 % of samples with BEA, FB1 and FB2 - 3 % of samples with BEA and OTA	Jurjevic *et al.* (2002)
FBs; ZEN	maize	- 40 % of samples with FB1 and ZEN	Domijan *et al.* (2005)
FBs; DON; ZEN; OTA	maize and maize products	- 57 % of samples with co-occurring mycotoxins - 38 % of samples with FBs, DON and ZEN	Kirincic *et al.* (2015)
	maize	- 40 % of samples with FB1, ZEN and OTA - 6 % of samples with FB1, FB2 and OTA	Domijan *et al.* (2005)
	maize	- high occurrence of DON and DON3G	Desmarchelier and Seefelder (2011)

Mycotoxin	Commodity	Observation	References
DON; DON derivates	maize and maize products	- high co-occurrence of DON, 3-ADON, 15-ADON and DON3G	De Boevre *et al.* (2012)
	maize	- consistent co-occurrence of DON and DON3G in all tested samples	Berthiller *et al.* (2009b)
	maize	- 50% of sample with DON + its acetylated and/or glycosylated derivates	Zachariasova *et al.*(2014)
DON; BEA	maize	- 38 % of sample with DON and BEA	Zachariasova *et al.* (2014)
DON; ZEN	maize and maize products	- 25 % of samples with DON and ZEN	Kirincic *et al.* (2015)
	maize	- 26 % of sample with DON and ZEN	Zachariasova *et al.* (2014)
DON; T2-HT2	maize and maize products	- high co-occurrence of DON and HT2	Cano-Sancho *et al.*(2012)
DON;NIV; T2-HT2	maize	- relatively high content of NIV, higher than for DON for same samples	Rasmussen *et al.* (2012)

Abbreviations: aflatoxins (AFs), fumonisins (FBs), fumonisin B1 (FB1), fumonisin B2 (FB2), deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON3G), 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), beauvericin (BEA), zearalenone (ZEN), T-2 toxin (T2), HT-2 toxin (HT2), nivalenol (NIV), ochratoxin A (OTA)

FIELD PREVENTION STRATEGIES FOR MAIZE MYCOTOXINS

Several research efforts have defined good agricultural practices (GAPs) to apply during pre-harvest stages, including: (i) farming systems, (ii) host resistance and hybrid selection, (iii) soil management, crop residues and crop rotations, (iv) irrigation, (v) pest and disease control, and (vi) biological control agents (BCAs) (Blandino et al., 2009a; Blandino et al., 2009b; Battilani et al., 2012).

Farming systems

Little information is available on fungal incidence in organic *versus* conventional farming of maize. Lazzaro *et al.,* (2015) demonstrated that *Fusarium* incidence was different between farming systems in Italian maize (20% in conventional production and 35% for organic production). However, *Aspergillus* incidence was not linked to the farming system but to weather conditions. Mycotoxin occurrence was not considered by Lazzaro *et al.,* (2015).
The most relevant agricultural factors that should be considered essential for integrated programmes to reduce *Aspergillus* and *Fusarium* toxins are outlined below, and are summarized in Supplementary Table S.1.

Host resistance and hybrid selection

Comprehensive knowledge of plant defense mechanisms may help to identify kernel resistance mechanisms, and assist the development of targeted and innovative approaches for breeding resistant crops (Alberts *et al.,* 2016). Plant breeding has been used as a tool to develop maize varieties resistant to abiotic and biotic stresses (Cary *et al.,* 2011; Lanubile *et al.,* 2011; Brown *et al.,* 2013; Farfan *et al.,* 2015; Lanubile *et al.,* 2017). These efforts have resulted in a number of germplasm releases. However, no maize hybrids were found to be completely resistant to fungal infection and/or mycotoxin contamination, because of the need to select for multiple traits and associated genes that contribute collectively to plant resistance. Resistance mechanisms are interconnected processes involving many gene products and transcriptional regulators, as well as host interactions with environmental factors, particularly, drought stress and high T (Jiang *et al.,* 2011). The molecular mechanisms underlying maize resistance have yet to be determined. Research has been devoted to understanding kernel resistant mechanisms at the transcriptional level, and to identify stress and/or defense

related genes induced during *A. flavus* infection in maize (Chen, *et al.,* 2001; Chen *et al.,* 2015). Microarray or proteomic studies have led to the discovery of many genes involved in maize resistance including several resistance-related quantitative trait loci (QTLs) (Kelley *et al.,* 2012; Brown *et al.,* 2013). Comparisons between the resistant and susceptible lines indicate differences in gene expression networks (Luo *et al.,* 2011). Several research outputs are available on plant-pathogen interactions and host resistance; these are promising starting points for future developments, but clear suggestions regarding hybrid selection, considered the best prevention tool, is not feasible.

Soil management, crop residues and crop rotation

Crop rotation and tillage are recommended practices to reduce inoculum of fungi on overwintering crop residues. Studies on the effects of these practices in maize show variable results, depending on the nature of the pathogen, the geographical location and the combinations with other strategies (Leslie and Logrieco, 2014). Under conditions of high T and low a_w, *A. flavus* becomes the dominant fungal species in the soil and produces abundant inoculum (Horn, 2003). *Fusarium* inoculum is always copious in crop residue in soil, irrespective of environmental conditions. Therefore, soil tillage is commonly considered to reduce inoculum availability. The effects of crop rotation are likely to be negligible, however, in areas with high prevalence of maize, because of long-distance air dispersal of *A. flavus* and *GF*sc (Munkvold, 2014).

Baliukoniene *et al.,* (2011) demonstrated that *F. verticillioides*, *F. proliferatum* and *F. subglutinans* survive for at least 630 d in maize stalk residues left on the soil surface or buried up to 30 cm deep. Under conventional tillage, the soil was contaminated with 7.0 ± 0.5 $\log_{10}$ CFU g^{-1} of fungal spores belonging to 17 genera of fungi. They identified *Fusarium* from 80% soil samples from conventional tillage. In contrast, the soil under no-tillage was contaminated with 13.5 ± 12.5 $\log_{10}$ CFU g^{-1} fungal spores. There is evidence that crop rotation has greater impacts on *F. graminearum* and *F. culmorum* and relative mycotoxins, especially DON and ZEN, rather than FB- and AF-producing fungi (Munkvold, 2014). This is consistent with splash dispersal of their inoculum. Besides affecting fungal population growth, soil conditions also influence plant root development. Crops with poorly developed root systems are more susceptible to water and nutritional stresses, and

consequently, are more susceptible to *Aspergillus* and *GF*sc infections. Adequate soil drainage to avoid drought stress, especially in clay soils, and adapting tillage strategies to soil conditions (Arino *et al.,* 2009; Blandino *et al.,* 2009a) may reduce fungal activity. Furthermore, crop rotation is applied to control maize pests. This practice is recommended in maize to reduce larval populations of western corn rootworm *(Diabrotica virgifera)* (Munkvold, 2014).

Irrigation

Maize has low tolerance to drought-stress, which is considered to be the most crucial factor promoting mycotoxin contamination, in addition to causing significant yield losses. Limited water availability predisposes plants to AF contamination (Battilani *et al.,* 2008; Abbas *et al.,* 2012; Torelli *et al.,* 2012; Damianidis *et al.,* 2018). For *A. flavus* infection, water stress is particularly critical during silk emergence and kernel ripening, so it is recommended to irrigate according to water needs taking into account also the evapo-transpiration precipitation (water balance). For geographical areas where water can be limiting, maize hybrids tolerant to water stress, in addition to early sowing, should be considered.

Data on FBs are less well defined compared with that for AFs. A field study by Arino *et al.* (2009) showed that drought stress during early maize reproductive growth was associated with increased risk for grain contamination with FBs due to *F. verticillioides*. However, the type of irrigation (flood or sprinkler) did not affect FB levels. Although the contribution of water stress to FB contamination is controversial, irrigation according to water needs to avoid drought stress to plants is still recommended, but avoiding excessive and prolonged irrigation close to the stage of milk ripening growth stage is important, as this could enhance FB accumulation (Blandino *et al.,* 2009a; Munkvold, 2014). Increases of DON concentration of up to 3.5 to 5-fold, caused by *F. graminearum,* were also documented by Oldenburg and Schittenhelm (2012) in kernels derived from limited watered plots compared to well-watered plots.

Pest and disease control

Several measures are applied against maize pests, including crop rotation, insecticides, fungicides and other chemical treatments, the use of resistant maize hybrids and biological control agents (BCAs), as well as monitoring and forecasting.

The use of insecticides reduces risk of mycotoxin contamination associated with insects (Folcher *et al.,* 2009). The links between insecticide use (mainly pyrethroids) for the control of ECB and reduction of FB contamination have frequently been described (Blandino *et al.,* 2009a; Blandino *et al.,* 2009b; Blandino *et al.,* 2009c; Folcher *et al.,* 2009; Mazzoni *et al.,* 2011; Folcher *et al.,* 2012). Studies of beneficial effects of combined use of insecticides and fungicides have provided equivocal results. Folcher *et al.* (2009) demonstrated no synergy between deltamethrin and tebuconazole. Efficacy for reducing FBs was 89.96% reduction from the insecticide treatment and 89.97% from insecticide + fungicide. Mazzoni *et al.,* (2011) demonstrated benefit from the combination deltamethrin + tebuconazole in reducing FB contamination, whereas no modification in AF content was observed after treatments. Content of FB1 decreased by 35% in plots treated with tebuconazole and by 56% with tebucoazole + deltamethrin.

Biological control agents (BCAs)

Several pre-harvest biological control systems have been developed for maize against *Aspergillus* spp. and *Fusarium* spp. These have used a variety of potential biocontrol agents (BCAs), including fungal and bacterial strains or atoxigenic fungal strains, as summarized in Table 2. Many microorganisms have been tested, but only *Trichoderma harzianum* (Nayaka *et al*., 2010) and *Clonostachys rosea* (Luongo *et al.,* 2005; Xue *et al*., 2014; Samsudin *et al*., 2017) have been studied under field conditions, and only atoxigenic *A. flavus* strains have been applied on large scale. Biological control of pathogenic *A. flavus* has been based on the use of atoxigenic isolates of this fungus, which act through competitive exclusion of AF-producers in the environment, and during crop tissue infection (Cotty and Bayman, 1993). The efficacy of this technique has been validated for control of AF contamination in maize. Two bio-pesticides with atoxigenic *A. flavus* active ingredients are registered for use on maize crops in the USA (Cotty, 2006), and several are available in the sub-Saharan Africa, grouped under AFLASAFE mark (Bandyopadhyay *et al.,* 2016). Atoxigenic *A. flavus* communities that are endemic to Italy have been identified, and their efficacy for reducing AF contamination by AF-producers has been demonstrated. One strain (MUCL 54911) displayed the greatest efficacy against several AF-producers (Mauro *et al.,* 2015), and was selected as the active ingredient in AF-X1, now under consideration for registration in Europe (Mauro *et al.,* 2018). To maximize efficacy for preventing aflatoxin contamination, the product should be adapted to the target crop and environment (Cotty, 2006), and the product should also be applied at the 5th leaf crop growth stage (Mauro *et al.,* 2015).

Far less field-based information is available on the effects of BCAs on FB-producing *Fusarium* spp. Results of bio-assays conducted under controlled conditions have demonstrated moderate suppression of toxigenic *F. verticillioides* and *F. proliferatum* strains using non-pathogenic *Fusarium* strains, including *F. equiseti* (Luongo *et al.*, 2005). Samsudin *et al.*, (2017) studied the effects of two BCAs, a fungus (*C. rosea*) and a gram-negative bacterium (BCA5), on growth rates of *F. verticillioides* (FV1), the relative expression of the FUM1 gene and FB1 production. The fungal antagonist reduced FB1 contamination on maize cobs by >70% at 25°C, and almost 60% at 30°C regardless of the maize ripening stage. For the bacterial antagonist, however, FB1 levels on maize cobs were significantly decreased only in some temperature/a_w treatments (25° C and a_w=0.976-0.958; 30° C and a_w=0.976). Abdallah *et al.*, (2018) demonstrated the capacity of two endophytic fungi (*Epicoccum nigrum* and *Sardoria fimicola*) to reduce ZEN amounts in maize under *in vitro* and *in planta* conditions. *Epicoccum nigrum* consistently reduced amounts of DON and 15-ADON. Some microorganisms have also been studied *in vitro* for their ability to inhibit spoiling *Aspergillus* spp. and *Fusarium* spp. species in maize feed and food products, and for use as natural post-harvest preserving agents (Table 2).

Table 2. Current information on reduction of mycotoxin-producing *Aspergillus* spp. and *Fusarium* spp., and mycotoxins production by biocontrol microorganisms *in vitro*, *in planta*, and in field trials in maize.

BCA(s)	Target fungal species	Type of assay	References
Pre-harvest			
Atoxigenic *A.flavus* strains	*A. flavus*	*In vitro* and in field	Cotty and Bayman (1993); Cotty (2006); Mauro *et al.* (2015); Bandyopadhyay *et al.*(2016); Mauro *et al.* (2018)
Trichoderma harzianum	*A. flavus*	In greenhouse and in field	Sivparsad and Laing (2016)

Streptomyces spp.	*A. flavus*	*In vitro*	Verheecke *et al.* (2016)
Bacillus megaterium	*A. flavus*	*In vitro*	Kong *et al.* (2014)
Bacillus subtilis (CW14)	*Aspergillus spp., Penicillium spp.*	*In vitro*	Shi *et al.* (2014)
Saccharomyces cerevisiae	*A. parasiticus*	*In vitro*	Armando *et al.* (2012)
Clonostachys rosea, Gram negative bacterium (BCA5)	*F. verticillioides*	*In vitro*	Samsudin *et al.* (2017)
Atoxigenic F.equiseti Clonostachys rosea, Epicoccum nigrum, Idriella bolleyi, Trichoderma harzianum, Trichoderma viride	*F. culmorum F. graminearum F. proliferatum F. verticillioides*	In field	Luongo *et al.* (2005)
Epicoccum nigrum	*F. graminearum*	*In vitro and in planta*	Abdallah *et al.* (2018)
Bacillus mojavensis (RRC101)	*F. verticillioides*	*In vitro*	Blacutt *et al.* (2016)
Bacillus spp., *Pseudomonas* spp. *Paenibacillus* spp.	*F. verticillioides*	*In planta*	Figueroa-López *et al.* (2016)
Trichoderma harzianum	*F. verticillioides*	*In vitro, in greehouse* and in field	Nayaka *et al.* (2010)
Clonostachys rosea	*F. graminearum*	In field	Xue *et al.* (2014)
Trichoderma asperellum	*F. graminearum*	*In vitro* and *in planta*	Yaqian *et al.* (2016)
Post-harvest			
Pichia anomala	*A. flavus*	*In vitro*	Tayel *et al.* (2013); Hua *et al.* (2014)
Lactobacillus plantarum	*A. flavus*	*In vitro*	Ahlberg *et al.* (2017)

Debaryomyces hansenii,BCS003	*Aspergillus spp., F. proliferatum, F. subglutinans*	*In vitro*	Medina-Cordova *et al.* (2016)
Lactobacillus plantarum MYS6	*F. proliferatum*	*In vitro*	Deepthi *et al.* (2016)
Lactobacillus delbrueckii *L. acidophilus* *L. sakei* *Pediococcus acidilactici* *Enterococcus faecalis*	*F. proliferatum*	*In vitro*	Khalil *et al.* (2013)

GRAIN HARVESTING AND DRYING

Late harvesting has major impacts on the levels of mycotoxins in maize grain, possibly due to high grain moisture levels and greater periods for fungal growth and toxin production (Munkvold, 2014). *Apergillus flavus* efficiently produces AFs when maize grain moisture content is less er than 28%. In this context, high T (>25°C) and a_w less than 0.95 have been suggested as thresholds above which AF accumulates rapidly (Giorni *et al.,* 2016). To reduce AF contamination, therefore, harvesting in hot and dry years should be carried out while avoiding very low moisture contents in maize grain, and limiting the time available for rapid growth of *A. flavus* and rapid synthesis of AFs. A working compromise for farmers would be to harvest at 22-24% grain moisture, but not at less than 20%.

Detrimental effects of a late harvesting are also confirmed in *Fusarium* spp. A study conducted on maize silage in Switzerland demonstrated that samples with high DON contents often came from fields harvested after September (Eckard *et al.,* 2011).

Moisture content of maize grain at harvest is commonly not low enough to guarantee safe storage, so the grain must be dried before storage commences (Bullerman and Bianchini, 2014). Drying is performed using heated air dryers. Many technologies, and different Ts and time combinations, can be applied for artificial drying of cereals. Treatments at 70°C for 24 h have been shown to be the

more effective for reducing the incidence and extent of fungal populations, than greater T and shorter exposure time (95°C for 9 h) (Giorni *et al.*, 2015). Grain should also be dried to less than 14% moisture content to be stored safely, with rapid reduction of moisture content during the first 24 h post-harvest. A final moisture content <13% is suggested when *A. flavus* is present (Channaiah and Maier, 2014).

POST-HARVEST GRAIN MANAGEMENT TO MINIMIZE RISKS OF MYCOTOXIN CONTAMINATION

Grain cleaning and grading

Pest attacks, harvesting and subsequent handling of maize grain can generate broken kernels, as well as contamination from soil and foreign materials which may be sources of mycotoxin contamination. Several physical processes are used for automated grain cleaning and grading (e.g. sieving, flotation, density segregation). Maize cleaning is commonly applied to remove powder and small kernel pieces, commonly the portions with the greatest mycotoxin contamination. Grading gained increased interest for improving grain lots to comply with legislated standards for processed products. Originally, grain grading machines were based on particle weight and size and used centrifugation and flotation in air flows. Contemporary grading machines are mainly based on optical sensors. Grading using UV light illumination for AF reduction is widely used, although mycotoxins can accumulate without visible symptoms and so pose limits to the use of optical sorting techniques (Karlovsky *et al.*, 2016).

Studies on the effectiveness of gain cleaning/grading processes have produced equivocal results, possibly due to the different initial levels of contamination of the raw materials tested (Pietri *et al.*, 2009), and because of differences between mycotoxins. Intact kernels were shown to contain approx. 10 times less FBs than broken maize kernels (Murphy *et al.*, 1993), and removal of broken kernels and other impurities from unprocessed maize reduced DON and ZEN by around 70–80 % (Trenholm *et al.*, 1991). For FB, however, contrasting results have been published. The cleaning step did not affect FB concentration from unprocessed and cleaned maize grain with low contamination (Generotti *et al.*, 2015), while a decrease of 45% was in medium-high contaminated grain (Fandohan *et al.*, 2005). Removal of fine material (approx. 10% by weight) in maize grain has been shown to reduce AF levels by 84% (Hu *et al.*, 2017).

Grain storage

After drying and cleaning, maize grain is placed in silos, for short or long periods, where it is prone to toxigenic fungal contamination and subsequent mycotoxin production, if conductive conditions occur. Air temperature, relative humidity and kernel moisture content have been identified as major storage factors influencing fungal activity and grain quality. Moderate T, kernel moisture less than 14% and dry environment have been demonstrated to limit *A. flavus* growth and subsequent AF contamination in stored maize (Giorni *et al.,* 2008). Monitoring of T and moisture has been suggested for early detection of fungal growth (Mason and Woloshuk, 2010), and this can be done using manual grain inspection for spoilage by moulds and other quality parameters, and measuring grain T. Both approaches, however, have inherent limitations: human sensory detection could be influenced by subjectivity errors caused by individual biases. Cables used to monitor T inside bulk grain bins detect changes only when spoiling grain mass is large enough to raise the T, and these changes must happen close to the sensors. Recent studies have examined the use of CO_2 production as an early indicator of levels of AFs (Garcia-Cela *et al.,* 2019) or FBs (Mylona *et al.,* 2012) in stored maize, and in other cereals (Mylona *et al.,* 2011; Martín Castaño *et al.,* 2017). These studies have shown CO_2 production and trends in the respiration rates, measured by Gas Chromatographic (GC) equipment, can be used as 'storability risk indices' to predict overall quality changes in stored grain.

Hermetic storage in silo bags is an alternative method to mitigate variations of environmental parameters and prevent fungal activity. No variations in AFs, FBs, DON, and OTA or in fungal contamination was observed in silo bags when dynamics of fungi and related mycotoxins were examined during maize storage (Gregori *et al.,* 2013).

Natural compounds with fungicidal or fungistatic activity may be useful for preventing fungal growth in stored maize (Bullerman and Bianchini, 2014; Caceres *et al.,* 2016). Different categories of plant-based compounds with bioactivity against a wide range of fungi have been identified as alternative agents, including antioxidants (Coma *et al.,* 2011; Azaiez *et al.,* 2013; De Lucca *et al.,* 2013; Thippeswamy *et al.,* 2013; Tracz *et al.,* 2016), phenolic compounds (Ferrochio *et al.,* 2013; Thippeswamy *et al.,* 2015), and essential oils (Da Gloria *et al.,* 2010; Matasyoh *et al.,* 2011; Elsamra *et al.,* 2012; Garcia *et al.,* 2012; Koc and Kara, 2014; Sahab *et al.,* 2014; Abhishek *et al.,* 2015; Kalagatur *et al.,* 2015; Liang *et al.,* 2015; Achugbu *et al.,* 2016; Kosegarten *et al.,* 2017; Sawai*et al.,* 2017) (see Table S.2). It is difficult to draw general conclusions from available information, due to the diversity of variables considered, including the fungal species and the types of compounds tested. Results have mostly been from small scale experiments, and efficacy in maize

storage trials remains to be tested and confirmed. Some general conclusions can be drawn, but results remain to confirmed in practical situations. Most studies have tested effects of particular compounds on fungal growth, whereas few have reported effects on mycotoxin reduction. The reported inhibition rates on AFs (Thippeswamy *et al.,* 2013; Liang *et al.,* 2015; Tracz *et al.,* 2016) and on FBs (Coma *et al.,* 2011; Elsamra *et al.,* 2012; Thippeswamy *et al.,* 2015) ranged from 30 to 100%. Eugenol (4-allyl-2-methoxyphenol) has been frequently reported as the active ingredient in the majority of the tested essential oils (eugenol concentration 34.7 - 78.4 %), highlighting the promise for this compound to reduce *Aspergilli* and *Fusaria* toxin production (Sahab *et al.,* 2014; Kalagatur *et al.,* 2015; Sawai *et al.,* 2017).

Grain processing

Food and feed processing can have affect initial content of mycotoxins in raw materials and these processes are here discussed individually.

Milling of maize grain does not destroy mycotoxins, but this process leads to redistribution of mycotoxins among mill fractions. Distribution of *Aspergillus* and *Fusarium* toxins in maize products after dry-milling has been investigated in several studies, showing similar patterns of distribution. Mycotoxin contaminations increase, compared to unprocessed maize grain, in bran, germ and fractions intended for animal feed (Coradi *et al.,* 2016), whereas they decrease in flaking grits and flour which are mainly destined to human consumption (Bullerman and Bianchini, 2014; Savi *et al.,* 2016). The distribution of *Fusarium* toxins (FBs, ZEN and DON) in dry-milled maize products has been assessed, and these results indicate that average mycotoxin content in meals and grits was reduced by 65-88% compared to the unprocessed grain (Reyneri *et al.,* 2004). A significant decrease (40%) in FB content from unprocessed maize to cornmeal semolina has also been demonstrated, whereas a significant increase in FB content has been found in middlings, commonly intended for feed production (Generotti *et al.,* 2015). In wet-milling, mycotoxins may be dissolved in the steep water and further redistributed. Forty to 50% of AFs were moved from corn grain into steep water during wet milling, where 28–38% of these mycotoxins remained in the fiber fraction, 11–17% in the gluten fraction, 6–11% in the germ, and only 1% in starch (Karlovsky *et al.,* 2016; Vanara *et al.,* 2018).

Thermal processing. Most mycotoxins are heat stable, but varying degrees of destruction can be achieved with the application of different time/T combinations. AFs have high decomposition Ts ranging from 237°C to 306°C, but all heat treatments (boiling, roasting, baking or steaming) have been reported to reduce foodstuff contamination (Jalili, 2015). Boiling maize grits reduced AF levels by

28%, while frying the boiled grits gave total reduction of 34-53% (Bullerman and Bianchini, 2014). Also, FBs are moderately stable compounds in high T, as a significant decrease in these compounds only occurs above 150-200°C, where thermal processing such as baking, frying, roasting or extruding are applied (Humpf and Voss, 2004; Mohanlall *et al.,* 2013). Bread baking has been shown to reduce concentrations of free FBs by 30-32% and concentrations of modified FBs by 10-19%. The differences in reduction of modified FBs were explained by the presence of proteins or starch capable of stabilizing the mycotoxins during baking (Bryła *et al.,* 2014). The effects of bread making on DON, T-2 and HT-2 toxin stability in naturally contaminated flour samples have been studied in wheat, but no data are available for maize derived products (Stadler *et al.,* 2018). Increases of DON after bread making have been reported, whereas the conjugated form as glucoside derivative DON3G (deoxynivalenol-3-glucoside) was reduced by approx. 50% after baking (Monaci *et al.,* 2013). In contrast, only 7.2% degradation of DON was recorded after baking at 100-250°C for 180 min (Numanoglu *et al.,* 2012).

Decreases in FB contents after thermal processing could be ascribed to the masking phenomena, as well as possible modifications of mycotoxin structure through interactions with other food components leading to the formation of conjugates (Falavigna *et al.,* 2012). Free and total FBs have also been shown to increase after heated drying, especially at 70°C for 24 h exposure. This evidence suggests possible retrogradation of starch, after heating, particularly for amylose, was closely related to modifications in detectable FBs (Giorni *et al.,* 2015).

Flaking and extrusion processes, obtained with high pressure and heating, have been recently reviewed (Jackson *et al.,* 2012; Bullerman and Bianchini, 2014). Several reports showed that FBs decreased after cornflake processing. About 60 to 70% of the initial amounts of FB1 and FB2 were lost during entire cycle of cornflake processing, with less than 30% losses occurring during the intermediate extrusion-cooking step (De Girolamo *et al.,* 2001). During extrusion cooking, the product is forced through metal tubes by rotating screws and is subjected to high T, high pressure, and severe shear. Extrusion usually causes decreases in mycotoxin concentrations. However, the effects on mycotoxin levels is probably influenced by the screw speed and T. Stability of FB1 in corn grits was affected by the extrusion parameters: up to 50% reduction in FB1 was measured when the grits were extruded at 160°C (Jackson *et al.,* 2012). The effects of extrusion on AF levels was also influenced by the presence or absence of additives, moisture content and T. Extrusion alone reduced AF content by 50–80%, and with addition of ammonia, either as hydroxide (0.7-1.0%) or as bicarbonate (0.4%), the decreases in AF levels

were greater than 95% (Jalili, 2015). Inclusion of sugar also altered the stability of FBs during extrusion processing (Castelo *et al.,* 2006). This was also the case for DON for which extrusion decomposed DON, which was more susceptible to extrusion than AFB1 (Cazzaniga *et al.,* 2001).

Traditional nixtamalization production of tortillas, the process of cooking in alkaline solution, is reduced initial total AFs by 60-65% and FBs by 80% (Schaarschmidt *et al.,* 2019). This was through physical removal during steeping and washing, and by degradation after application of elevated pH and high T. However, the reductions varied depending on cooking time T, steeping time, and initial toxin concentration in maize grain (Mendez-Albores *et al.,* 2014). The impacts of different nixtamalization processes on AF and FB concentrations was reviewed by Schaarschmidt *et al.* (2019). Besides reduction in the free parent forms, nixtamalization can also cause modification, and/or binding or release of matrix-associated mycotoxins, but their toxicity has yet to be evaluated (De Girolamo *et al.,* 2016).

Detoxification

Preventive actions are not effective for fully avoiding mycotoxin contamination, so detoxification methods may still be necessary to recover contaminated commodities. These include the use of physical processes, or chemical and biological additives. The efficacy of these processes in reducing AFB1 was reviewed by Rushing *et al.,* (2019). They reported a reduction range of AFB1 between 51 and 100% after thermal treatment at Ts between 150 and 200°C, and exposure times between 20 and 200 min. However, none of the reviewed studies were conducted on maize matrices, but were on other cereals (rice and wheat). Gbashi *et al.,* (2019) examined decontamination effects of heating on maize flour, and demonstrated that AFs (AFB1, AFB2, AFG1) were completely degraded at 217°C for 35 min. Heat treatment is a low cost and simple approach for mitigating the presence of mycotoxins. However, thermal stability of mycotoxins requires the use of high Ts and long exposure times, which result in a significant impact on grain quality factors.

Effects of UV or gamma irradiation have been reported in maize for AFB1 (Markov *et al.,* 2015) and FBs (Mansur *et al*., 2014). Reductions of AFB1 by radiation were reported to range between 60 and 90% (Markov *et al.,* 2015).

Chemical treatments have included acidification, ammonization and ozonation, the latter has shown a decontamination rate of AFB1 in maize of 88% (Luo *et al.,* 2014).

Microbial degradation of mycotoxins in less-toxic products has been examined. These biological treatments include inoculation with *Bacillus* (Oluwafemi *et al.,* 2010; Noah Badr *et al.,* 2017) or yeast species (Verheecke *et al.,* 2016), and botanical extracts or enzymes from different biological sources (Karlovsky *et al.,* 2016), with reported reductions in mycotoxins of 60-100%. However, all the described methods are remain experimental, and have yet to be considered as practical management strategies for mycotoxin detoxification.

MODELLING, AND EFFECTS OF CLIMATE CHANGE

Mechanistic models, using weather data as inputs, can predict mycotoxin contamination during the maize growing season and at harvest. They provide valuable support to crop management in a whole food chain view aimed at minimizing mycotoxin contamination. Mechanistic models are available for the prediction of AF and FB occurrence in maize crops, based on actual weather data (Battilani *et al.,* 2003; Maiorano *et al.,* 2009; Battilani *et al.,* 2013), but have not been developed for DON contamination. The impacts of cropping systems are yet to be included in these models. The models could be adapted for the post-harvest periods, but this has yet to be considered. Instead, risk maps have been drawn using historical meteorological data inputs to characterize the most common contamination in relevant geographic areas (Battilani and Camardo Leggieri, 2015).

Apart from seasonal prediction and risk maps, the interest in predictive models for mycotoxins contamination in crops is increasing to take account of climate change. At a global level, climate change is expected to have significant impacts on plant biogeography and fungal populations, with consequences on mycotoxin patterns, as confirmed with predictive approaches (Battilani *et al.,* 2016; van der Fels-Klerx *et al.,* 2016), and by field surveys in Europe (Piva *et al.,* 2006; Dobolyi *et al.,* 2013; Levic *et al.,* 2013). Uncertainties in climate conditions and extreme events have been stressed, and also described as crucial at farm levels (Camardo Leggieri *et al.,*2019), increasing the emerging risk of co-occurring mycotoxins. Predictive models have therefore become important, to address uncertainties and highlight risk conditions on a geographic basis. Predictive models are likely to be important tools in chain management for mycotoxin reduction as support for farmers, extension services and stakeholders. These willrationalize pre- and post-harvest crop and product management, and provide tools to policy makers for relevant strategic decisions.

CONCLUSIONS

This review has addressed *Aspergillus* and *Fusarium* species in maize, and provided an account of available strategies to mitigate the occurrence of AFs, FBs and DON in maize. Mycotoxin contamination with more than one congener, including modified mycotoxin forms, is an issue that needs further investigation, particularly regarding the consequences for human and animal health. A large body of literature exists on fungal growth and mycotoxin production, and on factors impacting plant-pathogen interactions. Research efforts to support the development of mycotoxin prevention strategies have resulted in sound mitigation methods, mainly at pre-harvest stages (Figure 3). Nevertheless, removal of mycotoxin contamination in maize cannot yet be foreseen, and further efforts are needed to increase the production of maize with mycotoxins below safe levels set by scientific advisory bodies. Key research areas that need further attention include:

i. Management of maize genetic resistance, with particular focus on effectiveness towards all mycotoxin producing fungi;
ii. Increased understanding of plant-pathogen interactions and plant defense mechanisms, including the role of mycotoxins in maize-fungi cross-talk;
iii. Extension of biocontrol to Fusaria and pest control as sustainable approaches for mycotoxin mitigation;
iv. Improvement of the performance of predictive models, including investigating the impacts of cropping systems and of co-occurring fungi on model predictions;
v. Prediction of future scenarios of mycotoxin occurrence as supporting tools for decision makers;
vi. Further development of alternative biological tools to be applied post-harvest, to improve safe storage or detoxification of contaminated grain and complete sustainable management of the maize value chain.

Harmonized methodologies for human and animal health risk assessment have been recently developed (EFSA, 2019). Such methodologies need to be applied to multiple mycotoxins, using available co-occurrence data and comparative toxicity metrics, to investigate the potential impacts on human and animal health of multiple mycotoxins, in a range of crops including maize.

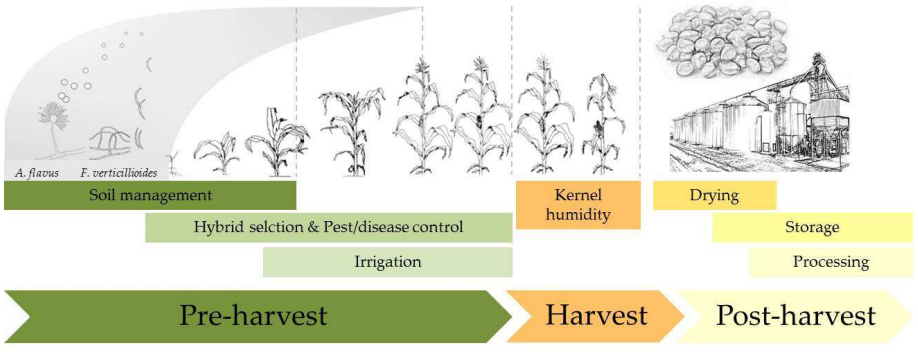

Figure 3. Crucial action in pre- and post-harvest management of maize to minimize mycotoxin contamination by *Aspergillus flavus* and *Fusarium vertcillioides*. Crop phenology is based on the BBCH scale edited by the Federal Biological Research Centre for Agriculture and Forestry.

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Table S.1: Current information on the main effect of agricultural practices on mycotoxin content in maize.

	AFs	FBs	DON	ZEN	Notes	References
Agricultural Practice						
Sowing/plant density		+	+		FB1 and FB2 occurrence was always significantly higher ($P<0.05$) with late (May) compared to early sowing date (March-April). In DON, the effect of early sowing was only significant in 2007 for the early hybrid, while the effect of high density (80,000 pt/ha) *vs* low density (65,000 pt/ha) differed over the three years according to the hybrid maturity.	Blandino *et al.*, 2009b
		+			Earlier planting consistently resulted in lower ear rot severity and FB1 contamination.	Parsons and Munkvold, 2012
		+			High plant density (80000 pt ha^{-1}), nitrogen fertilization and late sowing date (May 3 to May 16) increased FBs contamination (+ 133 %).	Blandino *et al.*, 2009a
		+			Maize fields subjected to dry planting contained significantly more FBs (1,004 ± 379 µg/kg) than those sown by wet planting (230 ±88 µg/kg) ($P<0.10$).	Arino *et al.*, 2009
Hybrid		-	+		FBs concentration did not differ significantly depending on the hybrid season length. A significant increased DON concentration in the kernels when associated with the use of late maturity hybrids.	Blandino *et al.*, 2009b
		+			Hybrid frequently had significant effects ($P\leq0.05$) on FB1 contamination.	Parsons and Munkvold, 2012

		-			The effect of hybrid maturity and the interactions between the independent variables (agricultural practices and hybrid) and random factors (year and site) were never significant.	Blandino *et al.*, 2009a
		+			The main role of fatty acids, with a higher FBs (FB1+FB2+FB3) contamination in hybrids showing a higher linoleic acid content and a higher masking action in hybrids with higher oleic to linoleic ratio.	Dall'Asta *et al.*, 2012
Soil management			-	-	In the soil under no-tillage, contamination with fungal spores was 92.9 % higher compared to the soil under conventional tillage. DON and ZEN content varied but it was not considerably influenced by the different tillage systems applied.	Baliukoniene *et al.*, 2011
Irrigation	+				Drought level represented by weekly-ARID (Agricultural Reference Index for Drought) values before and after mid-silk is a significant predictor (p-value < 0.10) for AF contamination risk.	Damianidis *et al.*, 2018
	+				Aridity indexes based on meteorological data confirmed the influence of drought conditions on AFB1 synthesis.	Battilani *et al.*, 2008
	+				AF levels averaged over a period of three years from 33.5 ± 12.0 $\mu g \cdot kg^{-1}$ for non-irrigated, 29.3 ± 11.4 $\mu g \cdot kg^{-1}$ for moderately irrigated and 24.2 ± 8.5 $\mu g \cdot kg^{-1}$ for well-irrigated plots.	Abbas *et al.*, 2012
		+			Irrigation significantly affected the level of FB contamination ($P<0.05$)	Torelli *et al.*, 2012
		-			The type of irrigation had no distinct effect ($P > 0.10$) on the FB levels, which were 508 ± 219 and 555 ± 275 µg/kg in fields with flood and sprinkler irrigation systems, respectively.	Arino *et al.*, 2009

			+		3.5 - to 5-times higher DON concentration in kernels at limited- than well-watered conditions: 380 compared with 75 µg/kg.	Oldenburg and Schittenhelm, 2012
Pesticides		+	-		The insecticide treatments (alpha-cypermethrin) against second-generation ECB larvae significantly reduce the FBs contamination but did not significantly reduce the DON contamination.	Blandino *et al.*, 2009b
		+			Insecticide treatments (active ingredient: alpha-cypermethrin) reduced (-54 %) the FB occurrence compared to the untreated control.	Blandino *et al.*, 2009a
	-	+			ECB control (deltamethrin) and tebuconazole applied at BBCH67. FB1 varied from 6767 µg/kg in the unsprayed plot to 4429 (-35 %) and 3013 (-56 %) µg/kg in respectively the plot treated with tebuconazole and with the addition of deltamethrin.	Mazzoni *et al.*, 2011
		+		+	The efficacy of ECB control was evaluated at 89.96 % for FBs with insecticide (deltamethrin) and 89.97 % with insecticide+fungicide (deltamethrin and tebuconazole). The efficacy was evaluated at 85.40 % for ZEN with insecticide and 82.10 % with insecticide+fungicide.	Folcher *et al.*, 2009
			+		Lambdacyhalothrin or deltamethrin (20 g ha^{-1}). DON levels were highly significantly affected by insecticide treatment ($F_{1,88}$=35.925; P <10^{-4}). On average, DON levels were significantly lower (151.73 µg/kg) in treated maize than in the control (849.04 µg/kg).	Folcher *et al.*, 2012
		+			Insecticides: A mixture of chlorpyrifos and cypermethrin applied at 0.450 and 0.045 kg ha^{-1} respectively. A significant effect (P < 0.001) of insecticide application timing on fumonisin occurrence in maize kernels was observed. Efficacy of the best application timing to control fumonisin occurrence was 73 % in 2006 and 84 % in 2007.	Blandino *et al.*, 2009c

- the agricultural practice has an impact (+); the agricultural practice does not have an impact (-)
- Abbreviations: aflatoxins (AFs), European corn borer (ECB), fumonisins (FBs), fumonisin B1 (FB1), fumonisin B2 (FB2), fumonisin B3 (FB3), deoxynivalenol (DON), zearalenone (ZEN)

Table S.2: Current information on reduction of mycotoxin-producing *Aspergillus* spp. and *Fusarium* spp. growth and mycotoxin production by plant-produced compounds.

Plant (compound)	Target fungal species	Type of assay	Reduction	References
Essential oils (EOs)				
Eucalyptus grandis, *E. staigeiriana*, *E. citriodora*, and the hybrid *E. grandis* x *E. urophylla*	*A. flavus* *A. parasiticus*	*In vitro*	*E. staigeiriana* showed the best potential on fungal growth control. The major active ingredients were limonene and geranial. The effect on mycotoxin production was not tested.	Da Gloria *et al.*, 2010
Cymbopogon citratus	*A. flavus* *A. parasiticus* *A. ochraceus* *A.niger* *A. fumigatus*	*In vitro*	The antifungal activity tests showed that the oil was active against all the five *Aspergillus* species, and the minimum inhibitory concentration (MIC) of the oil ranged from 15 to 118 mg/ml. The MIC ranged from 15 to 118 mg/ml. The major active ingredients were geranial, neral, myrecene and geraniol. The effect on mycotoxin production was not tested.	Matasyoh *et al.*, 2011

Thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), and laurel (*Laurus nobilis*)	*A. flavus* *A. parasiticus*	*In vitro/in vivo*	Thyme showed the highest inhibition on *A. parasiticus* growth (39 mm diameter of inhibition zone), followed by rosemary (15 mm) and laurel (10 mm).	Koc and Kara, 2014
Clove (*Syzygium aromaticum*) and vatica (*Vatica diospyroides*)	*A. flavus*	*In vitro/in vivo*	Clove showed 84.7 % inhibition on conidial germination of *A. flavus* at 100 µL L^{-1}, and complete inhibition of disease infection on maize seeds at 10 µL L^{-1}. Vatica completely inhibited growth, sporulation, conidial germination, and disease infection of *A. flavus* both *in vitro* and on maize seeds at 50 µL L^{-1}. The main active ingredients were eugenol (62.4 %) and benzyl acetate (48.8 %) for clove and vatica oil respectively. The effect on mycotoxin production was not tested.	Sawai *et al.*, 2017
Cinnamon (*Cinnamomum verum*) essential oil (CEO)	*A. flavus*	*In vitro*	*A. flavus* growth rate diminished and lag time increased as the concentration of CEO increased. The major active ingredients was cinnamaldehyde.	Kosegarten *et al.*, 2017
Cinnamaldehyde, citral and eugenol	*A. flavus*	*In vitro*	*A.flavus* growth and AFB1 production were completely inhibited by 0.80 mmol/L of cinnamaldehyde and 2.80 mmol/L of citral. At lower concentration, cinnamaldehyde, eugenol, and citral significantly reduced AFB1 production with	Liang *et al.*, 2015

			inhibition rate of 68.9 %, 95.4 %, and 41.8 %, respectively, while no effect on fungal growth.	
Ocimum sanctum essential oil (OSEO)	*F. graminearum*	*In vitro*	MIC and minimum fungicidal concentration of OSEO were 1250 and 1800 µg/mL, respectively. ZEN concentration was insignificant at 1500 µg/mL concentration. The main active ingredients was eugenol (34.7 %).	Kalagatur *et al.,* 2015
Rocket seeds (*Eruca sativa*), rosemary (*Rosmarinus officinalis*) and tea tree (*Melaleuca alternifolia*)	*F. graminearum* *F. avenaceum* *F. semitectum* *F. solani* *F. oxysporum*	*In vitro*	IC50 and MIC ranged from 0.044 to 0.049 % and 0.087 to 1.00 % for rocket essential oil, and from 0.049 to 0.282 % and 0.455 to 0.616 % for rosemary essential oil, and from 0.043 to 0.170 % and 0.192 to 0.361 % for tea tree essential oil respectively.	Sahab *et al.,* 2014
Clove (*Eugenia caryophyllata*), thyme (*Thymus vulgaris*) and black cumin (*Nigella sativa*)	*A. flavus* *F. verticillioides*	*In vitro*	Clove significantly decreased the growth of both tested fungi at all tested concentrations (0.1, 0.5 and 1%); it also resulted in AFs (45.47 %) and FBs (33.29 %) reduction. Black cumin (1 and 2 %) was effective only in suppressing the growth of *A. flavus.* The main active ingredient in clove oil was eugenol (78.41 %).	Elsamra *et al.,* 2012
Solanum torvum (Torvoside K)	*A. flavus* *F. verticillioides*	*In vitro/in vivo*	MICs ranged from 31.25 to 250 µg/ml^{-1}, for *A. flavus* and *F. verticillioides* respectively.	Abhishek *et al.,* 2015
Garcinia kolakola and *Azadirachta indica*	*A. flavus,* *A. parasiticus,*	*In vitro*	Inhibition growth was higher by *G. kola* for both fungi, respectively 77.5 % for *A.flavus* and 54.8 %	Achugbu *et al.,* 2016

			for *A.parasiticus*, and lowest by *A.indica,* 35.1 % and 30.5 % respectively.	
Equisetum arvense and *Stevia rebaudiana*	*A. flavus F.verticillioides*	*In vitro*	Inhibition of growth for both fungi was significant (>99 % inhibition at 0.95 a_w). AFB1 and FB1 presence were not significantly affected.	Garcia *et al.,* 2012
Antioxidants/Phenolic compounds				
2-hydroxy-4methoxybenzaldehyde (HMB) from *Decalepis hamiltonii*	*F. verticillioides*	*In vitro/in vivo*	Dose-dependent strong inhibitory activity with MIC value of 100 µg/mL, FB1 production was completely inhibited at 400 mg/L under *in vitro* and 750 mg/kg under *in vivo*.	Thippeswamy *et al.,* 2015
Allyl (AITC), phenyl (PITC) and benzyl isothiocyanates (BITC) from cruciferous vegetables	*F. verticillioides*	*In vitro*	The mean reduction of FB2 was 84.9 %.	Azaiez *et al.,* 2013
Allyl isothiocyanate (AITC) from brassica plants	*A. parasiticus F. tricinctum F. verticillioides Alternaria alternata F. graminearum*	*In vitro*	AITC treatments at 50, 100 and 500 mL/L inhibited visual growth of all fungal species and kept the production of 12 mycotoxins at undetectable levels (eg.AFB1 at 72.08 ± 12.70 mg/kg of corn; AFB2 at 2.14 ± 0.34 mg/kg)	Tracz *et al.,* 2016
Ferulic acid	*F. verticillioides F. proliferatum*	*In vitro*	The lag phase significantly decreased for both moulds ($p \leq 0.001$). However, 10 mM ferulic acid	Ferrochio *et al.,* 2013

			significantly increased ($p \leq 0.001$) fumonisin production.	
Tetra-hydro-curcuminoids (THCs) from natural curcuminoids	*F. proliferatum*	*In vitro*	Inhibition percentage of fungal growth reached 70 % at 13.4 μmol ml^{-1} concentration of THCs. FB1 reduction ranged between 31-37 %.	Coma *et al.*,2011
Budmunchiamine A (BUA) isolated from *Albizia amara* Pithecolobine (PI) isolated from *Albizia saman*	*A. flavus*	*In vitro*	Inhibitory effect on both *A. flavus* growth and AFB1 production by BUA and PI at concentration of 1 mg/mL. MIC ranged from 6.8 to 19.6 mm and 0.015–0.5 mg/mL,respectively for BUA and PI.	Thippeswamy *et al.*, 2013
Trans-2-hexenal (T2H)	*A. flavus*		Significant reduction of fungal populations. Absence of AFB1 in almost all sets of experiment.	De Lucca *et al.*, 2013

- Abbreviations: aflatoxins (AFs), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), fumonisins (FBs), fumonisin B1 (FB1), fumonisin B2 (FB2), inhibitory concentration (IC), inhibitory concentration at 50 % (IC50), minimum inhibitory concentration (MIC), zearalenone (ZEN).

Chapter II - Occurrence and co-occurrence of mycotoxins in cereal-based feed and food

Roberta Palumbo[1], Alfonso Crisci [2], Armando Venâncio [3], José Cortiñas Abrahantes[4], Jean-Lou Dorne [4], Paola Battilani [1] and Piero Toscano [2]

[1] Department of Sustainable Crop Production (DI.PRO.VE.S.), Faculty of Agriculture, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29100 Piacenza, Italy
[2] National Research Council, Institute of BioEconomy (IBE), 50145 Florence, Italy
[3] Centre of Biological Engineering (CEB), University of Minho, 4710-057 Braga, Portugal
[4] European Food Safety Authority (EFSA), 43126 Parma, Italy

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SUMMARY

Dietary (co)-exposure to mycotoxins is associated with human and animal health concerns as well as economic losses. This study aims to give a data-based insight from the scientific literature on the (co-)occurrence of mycotoxins (i.e., parent and modified forms) in European core cereals, and to estimate potential patterns of co-exposure in humans and animals. Mycotoxins were mainly reported in wheat and maize showing the highest concentrations of fumonisins (FBs), deoxynivalenol (DON), aflatoxins (AFs), and zearalenone (ZEN). The maximum concentrations of FB_1+FB_2 were reported in maize both in feed and food and were above legal maximum levels (MLs). Similar results were observed in DON-food, whose max concentrations in wheat, barley, maize, and oat exceeded the MLs. Co-occurrence was reported in 54.9% of total records, meaning that they were co-contaminated with at least two mycotoxins. In the context of parental mycotoxins, co-occurrence of DON was frequently observed with FBs in maize and ZEN in wheat; DON + NIV and DON + T2/HT2 were frequently reported in barley and oat, respectively. Apart from the occurrence of ZEN and its phase I and phase II modified forms, only a limited number of quantified data were available for other modified forms; i.e., mainly the acetyl derivatives of DON. Data gaps are highlighted together with the need for monitoring studies on multiple mycotoxins to identify co-occurrence patterns for parent mycotoxins, metabolites, and their modified forms.

Keywords: modified mycotoxins; fumonisin; aflatoxin; deoxynivalenol; maize; wheat; oat; barley; rice; extensive literature search

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by different genera of filamentous fungi that infect susceptible plants throughout the world (Gruber-Dorninger et al., 2019; Ingenbleek et al., 2019). These toxins are low molecular weight and very stable compounds likely to contaminate dietary staple foods, particularly cereals, along the entire production chain, especially under conductive pre- and post-harvest conditions. Crops may be infected with multiple species of mycotoxigenic fungi, and most fungal strains produce more than one type of mycotoxin. Therefore, co-contamination of agricultural products with multiple mycotoxins is frequently observed and recently emphasized (Grenier and Oswald, 2011; EFSA, 2017a, b, 2018). When raw materials are mixed to produce feed or processed into food, mycotoxin co-occurrence becomes even more likely. Although potential interventions to prevent field outbreaks have been considered in several crops worldwide (Torres et al., 2014; Gonçalves et al., 2019a, b; Torres et al., 2019; Palumbo et al., 2020), mycotoxins still represent an important public health and economic burden.

To date over 400 different mycotoxins have been identified with different chemical structures and properties, produced by a range of different fungal species. Among them, there are well characterized groups of mycotoxins such as aflatoxins (AFs), fumonisins (FBs), type A trichothecenes (e.g., T-2 and HT-2 toxin), type B trichothecenes (e.g., deoxynivalenol (DON), nivalenol (NIV)), zearalenone (ZEN), ochratoxin A (OTA), patulin (PAT), ergot alkaloids (EAs), as well as emerging toxins namely citrinin (CIT) and enniatins (ENNs). Of note, many structurally related congeners, defined as modified mycotoxins, are generated by plant, fungi metabolism, or food processing, and coexist with their native forms (Rychlik et al., 2014). As a consequence of their complex and variable chemical structure and ubiquitous presence, humans and animals can be potentially exposed to single or multiple mycotoxins through the consumption of contaminated diets.

Mycotoxins are well established to have a number of health impacts both in humans and animals. Depending on the quantities consumed, mycotoxins and their metabolites are associated with severe acute poisoning, including death, and chronic adverse health effects. The toxicity of several mycotoxins has been demonstrated for single compounds. Aflatoxin B_1 (AFB_1) was classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1), and recognized as one of the most potent liver genotoxic carcinogens. Fumonisins B_1 and B_2 (FB_1, FB_2) and OTA were classified in Group 2B, compounds

considered carcinogenic to animals and possibly carcinogenic to humans (IARC, 2012). IARC recently also associated AFs and FBs dietary exposure with high levels of stunting and growth impairment in children.

In addition, interaction effects (i.e., additive, synergistic, or antagonistic) have also been associated with the co-exposure to multi-mycotoxin. However, in the peer-reviewed literature there are still limited papers addressing toxicokinetics (TK) aspects after concurrent exposure to mycotoxins in living organisms (Fremy et al., 2019; Steinkellner et al., 2019; Gkrillas et al., 2020).

The effect of feed-borne mycotoxins on food-producing animal performance represents an economic problem for farmers; reduced growth, decreased egg and milk production, lower reproductive efficiency, and increased susceptibility to stress are all consequences of mycotoxin exposure. Moreover, consumers are potentially also exposed indirectly, due to the contamination in foods of animal origin due to carry-over (i.e., milk, eggs, etc.).

Multiple mycotoxins in feed and food have been recognized by European regulatory bodies as emerging risks in food safety and security with regards to animal and human health. Efforts to reduce human and animal exposure to mycotoxins resulted in the establishment of regulatory limits and monitoring programs worldwide. Maximum permitted levels (MLs) or guidance of safety levels have been provided in different countries. European legislation protects consumers by setting legal MLs for the main classes of mycotoxins in several core commodities intended for food and feed, like cereals, nuts, fruits, and derived products, including milk (European Commission, 2011, 2013, 2016). However, the current MLs do not consider the exposure to multiple mycotoxins and they are either based on the risk assessment of a single compound or on their sum, like the cases of AFs and FBs. According to the European Commission Regulation 1881/2006, and subsequent amendments, the MLs for AFs in cereals intended for direct human consumption is set to 2 μg/kg of AFB_1 and 4 μg/kg of the total sum of AFB_1, AFB_2, AFG_1, and AFG_2; whereas, the MLs for the sum of FB_1 and FB_2 is set to 1000 μg/kg in maize intended for direct human consumption, and 4000 μg/kg in unprocessed maize (European Commission, 2006b, 2010). In addition, guidance values for the sum of FB_1 and FB_2, and for DON have been recommended in products intended for animal feed in the EU (European Commission, 2006a).

The conventional exposure assessment paradigm of groups of populations to single mycotoxins utilizes consumption and occurrence data to derive exposure scenarios. In the context of multi-mycotoxins, a rationale way to perform risk assessments is by establishing priorities based either on the realistic frequency of

the co-occurring mycotoxins or by considering the potency of the combined toxic effect.

Therefore, monitoring mycotoxin co-occurrence enables identifying the most prevalent mycotoxin mixtures and, consequently, can help to prioritize research efforts. Thus, the aim of this paper is to provide a literature and data-driven insight on the presence of mycotoxins in cereal-derived feed and food commodities in Europe, and their natural co-occurrence.

2. MATERIALS AND METHODS

2.1. Data Collection and Data Extraction

An Extensive Literature Search (ELS) was undertaken in order to collect available papers in scientific literature on the (co-)occurrence of mycotoxins in core cereals, including maize, wheat, barley, oat, rice, rye, and sorghum from 2010 to 2018, and it was focused on the need of exposure calculations. When necessary, ad hoc searches with extended timeframes (up to 2000) were undertaken, as in the case of maize and sorghum. Mycotoxins with major public health and economic interest were included in the searching criteria, including those regulated at the European level and their modified forms, plus some emerging mycotoxins. Starting from a substantial initial number of 13,026 papers, the screening process resulted in a selection of 206 papers, which were used for data extraction. The following represents the flowchart associated with the selection of studies relevant to the aim of this study (Figure 1).

Since the collection of these data was meant to estimate dietary exposure of humans and animals in Europe, attention was paid to EU data, although the information on the origin of non-EU imported commodities was stored.

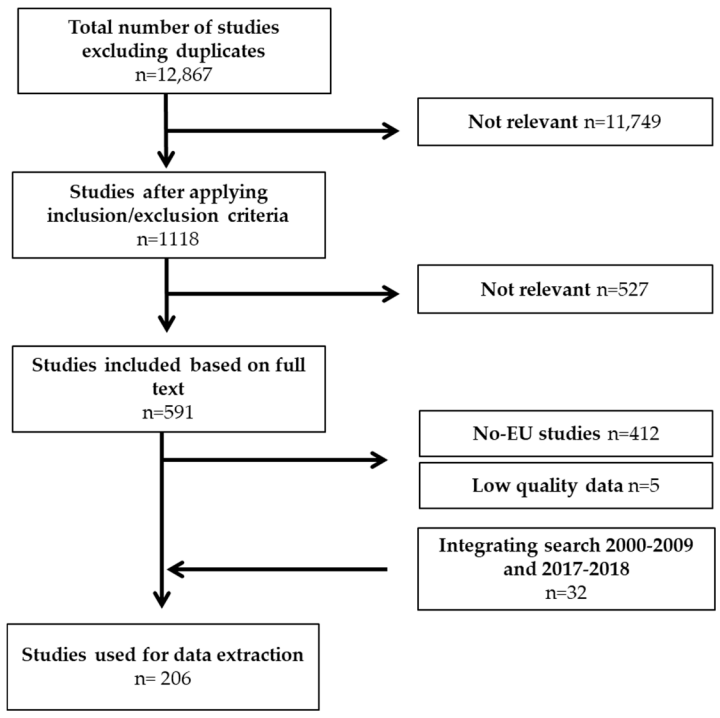

Figure 1. Flow chart of the extensive literature search performed.

2.2. Development of a Structured Database on Occurrence and Co-Occurence of Mycotoxins

A database on mycotoxins occurrence/co-occurrence was structured according to the European Food Safety Authority (EFSA) Standard Sample Description version 2 (SSD2) standard (EFSA, 2013). The SSD2 data model was used to support reporting countries in data submissions to the EFSA and structured to collect analytical results at the sample level. In our study, the standard data model was adapted to aggregate data, which is the way authors commonly report occurrence data in the literature. However, when the co-occurrence data were reported at the sample level, a univocal identification number (ID) was assigned to each specific sample. A comprehensive description of the individual data elements of the SSD2-based data model is provided in supplementary materials.

2.3. Data Analysis

General qualitative and quantitative descriptions of the ELS records were conducted providing an insight of both occurrence and co-occurrence of mycotoxins in EU countries. Descriptive statistics for concentrations of the most frequently occurring mycotoxins and their modified forms in cereal- based feed and food, as well as for studies that do not specify feed or food, were derived. A qualitative score was implemented for occurrence data while frequency and multinomial distribution analysis were performed for co-occurrence data. The data model and data analysis were designed and performed in a R environment (R Core Team, 2019), respectively. All data, functions, and codes are currently available on the MYCHIF project repository (Toscano, 2019).

2.4. Analysis of Occurrence Data

The database for the occurrence and co-occurrence data of mycotoxins in cereals includes 12 crop aggregations: barley, buckwheat, cereals, maize, oat, rice, rye, sorghum, spelt, triticale, wheat, and others (millet and soy). The authors noted that most often, in the case of mixed cereal grains- based commodities, the main ingredients were not indicated. For this reason, "cereals" were kept as one commodity category, intended as mixed cereals. Occurrence data for each mycotoxin, stratified by crop, were extracted and analyzed. Only records reporting concentration values (data at sample level) or mean values (aggregate data) were extracted. Values lower than the limit of detection (LOD) or lower than the limit of quantification (LOQ) were not included in the analysis, but tracked (<LOD = −1; <LOQ = −2) for further processing. Non-linear regression analysis was applied to characterize the type of distribution that best reflects each mycotoxin crop dataset block and to build a reliable reference exposure distribution that can be subsequently used for risk assessments. Weibull, gamma, lognormal, and normal distributions were tested for each data block and the benchmark with empirical data was characterized using the following:

-histogram and theoretical densities plot
-empirical and theoretical Cumulative Distribution Function (CDF) plot
-Quantile-Quantile (Q-Q) plot
-Probability-Probability (P-P) plot

To facilitate the visualization of the quality and quantity of the extracted datasets, measuring the strength of backward bibliographical context, a general scaled index based on 7 distinct scores, named $Score_{gen}$, was implemented as the sum of 7 partial sub-indices defined below:

$$\begin{aligned} Score_{gen} = {} & Score_{numerosity} + Score_{validity} + CV_{score} + P_{sampleSize} \\ & + P_{agePaper} + P_{bibIntensity} + P_{haveBounds} \end{aligned} \quad (1)$$

where Score numerosity refers to data availability (i.e., papers with at least 25 sample data were marked as 1); Score validity refers to the percentage of good data available (i.e., normalized mean of valid data given by a single paper); CV score refers to the coefficient of variation of toxin concentration calculated in records considered; P sampleSize refers to the total number of samples in all the records considered with at least 5 valid data; P agePaper refers to the age (years from the publication) of papers (i.e., normalized mean age of paper); P bibIntensity refers to bibliography intensity (i.e., normalized records of unique paper); and P haveBounds refers to records that provide also statistical information as range (i.e., Min/Max values). Each sub-index is based on data normalized in the range 0–1.

For a general view of $Score_{gen}$ index and all sub-indices corresponding to each combination of mycotoxin and crop, heatmap plots were then produced.

2.5. Analysis of Co-Occurrence Data

The number of co-occurrence cases for each crop was extracted for 2 or more mycotoxins on the same sample based on the data description in each individual publication extracted from the ELS (identified as co-occurrence = 1 in the database). From all data extracted, the resulting 4 crops (maize, wheat, barley and oat), 6 main co-occurring mycotoxins and their modified forms (AFs, DON, FBs, NIV, T2+HT2, and ZEN) provided data for a more detailed analysis. Soft wheat and durum wheat were aggregated only for data analysis of co-occurrence. Finally, average concentrations and the relative frequency of co-occurrence were calculated for each crop aggregation and co-occurrence pattern.

In the context of co-occurrence of mycotoxin native forms, the frequency in which a mycotoxin was reported alone (i.e., AFs and FBs) or in combination with others was recorded, allowing the identification of patterns of co-occurrence and their frequency for each dataset. The former was used to fit a multinomial model to estimate the probability of each mycotoxin present in a food or feed sample. Estimation of such probability was performed using a multinomial model using

frequencies of each combination of mycotoxin which was then simulated to estimate potential co-occurrence based on the observed patterns reported.

3. RESULTS

A total number of 8406 records and 1,440,646 samples were collected. The vast majority of the studies reported data from more than one cereal, and the most studied crops were found to be wheat (34%), maize (28%), barley (10%), oat (9%), and rice (6%) (Table 1). Buckwheat, rye, triticale, sorghum, spelt, and others (millet + soy) account altogether for 7%, with rye being the most studied. Furthermore, "cereals," accounted for 6% of total records.

Table 1. Total number of records per crop with specification on the number of records below the limit of detection, the limit of quantification, and co-occurrence studies.

Crop/Aggregation	N of Records [1]	<LOD [2]	<LOQ [3]	N of Co-Occ Studies [4]	N of Co-Occ Records [5]
Barley	865	140	109	17	330
Buckwheat	6	3	0	1	4
Cereals	463	189	61	12	223
Maize	2362	1055	66	27	1443
Oat	740	150	81	14	374
Rice	520	297	26	8	343
Rye	236	75	14	10	111
Sorghum	101	62	9	2	51
Spelt	83	26	1	3	61
Triticale	127	48	0	3	13
Wheat	2860	1252	142	43	1646
Others [6]	43	32	0	3	13
All	8406	3329	509	482	4612

[1] Total number of records; [2] Records reported as below the limit of detection; [3] Records reported as below the limit of quantification; [4] Number of co-occurrence studies; [5] Number of co-occurrence records; [6] Millet and soy.

Overall, data available were classified as referring to feed (2225 records), food (4104 records), feed and food (42 records), and cereals with no defined use (2035 records). The most frequently occurring mycotoxins and modified forms (i.e., number of records above twenty) in feed, food, and cereals with no defined use are displayed in Figure 2, Figure 3, and Figure 4, respectively.

Sample origins were not always reported for European countries, even if the analyses were performed in Europe, and these included a limited number of samples originating from Africa, Asia, and South America (n = 590 records of which 48 records as mix from different continents), namely rice (34.2%), wheat (21.9%), maize (15.8%), sorghum (13.0%), barley (3.9%), cereals (3.7%), rye (3.6%), oat (3.1%), and soy (0.8%).

Retrieved papers covered the period 2000–2018 with the majority of records distributed between 2010–2017, and the limited number of papers for the year 2018 is partly due to the limited span of the ELS for that year (i.e., last access in June 2018) (Figure 5).

The proportion of left censored data (LCD), intended as results below LOD (non-detected analytes) or below LOQ (detected but non-quantified analytes), ranged from 39.6% (<LOD) to 6.0% (<LOQ) (Table 1). Since these data were used for dietary exposure assessments in humans, these were treated by the substitution method (WHO, 2009; EFSA, 2010) so that (i) at the lower-bound (LB) all results reported as lower than the LOD were set to zero and to the numerical value of the LOD for results reported as lower than the LOQ; (ii) at the upper-bound (UB), the results below the LOD were set to the numerical value of the LOD and to the value of the LOQ for results below the LOQ.

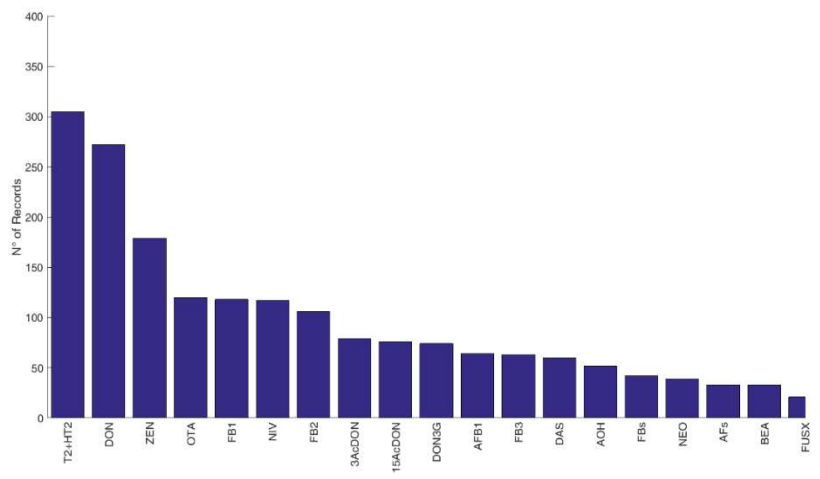

Figure 2. Frequencies of reported mycotoxins and secondary metabolites in feed in Europe. The figure displays the compounds with a number of records above twenty. $N > 20$: T2: T-2 toxin, HT2: HT-2 toxin, DON: deoxynivalenol, ZEN: zearalenone, OTA: ochratoxin A, FB1: fumonisin B1, NIV: nivalenol, FB2: fumonisin B2, 3Ac-DON: 3-

acetyldeoxynivalenol, 15Ac-DON: 15acetyldeoxynivalenol, DON3G: deoxynivalenol-3-glucoside, AFB1: aflatoxin B1, FB3: fumonisin B3, DAS: diacetoxyscirpenol, AOH: alternariol, FBs: total fumonisins, NEO: neosolaniol, AFs: total aflatoxins, BEA: beauvericin, FUS-X: fusarenon-X. $N < 20$ (not reported in the figure): HT2-3Glc: HT-2 toxin-3-diglucoside, T2-3Glc: T-2 toxin-3-diglucoside, AFB2: aflatoxin B2, AFG1: aflatoxin G1, AFG2: aflatoxin G2, α-ZEL: α-zearalenol, FB1+FB2: fumonisin B1 + fumonisin B2, AME: alternariol monomethyl ether, STO: scirpentriol, ALTERNARIA: alternaria toxins, β-ZEL: β-zearalenol, STC: sterigmatocystin, CIT: citrinin, ENB: enniatin B, MAS: monoacetoxyscirpenol, T2-tetraol: T2 tetraol, T2-triol: T2 triol, ENA: enniatin A, ENA1: enniatin A1, ENB1: enniatin B1, ENB2: enniatin B2, ALT: altenuene, Ergocornine, Ergocristine, Ergocryptine, AND A: andrastin A, αZEL14G: α-zearalenol-14-glucoside, Marcfortine A, MON: moniliformin, NIV3G: nivalenol-3-glucoside, ROQC: Roquefortine C, β-ZEL14G: β-zearalenol-14-glucoside, TeA: tenuazonic acid, ZEN14G: zearalenone-14-glucoside, ZEN14S: zearalenone-14-sulfate, ZEN16G: zearalenone-16-glucoside.

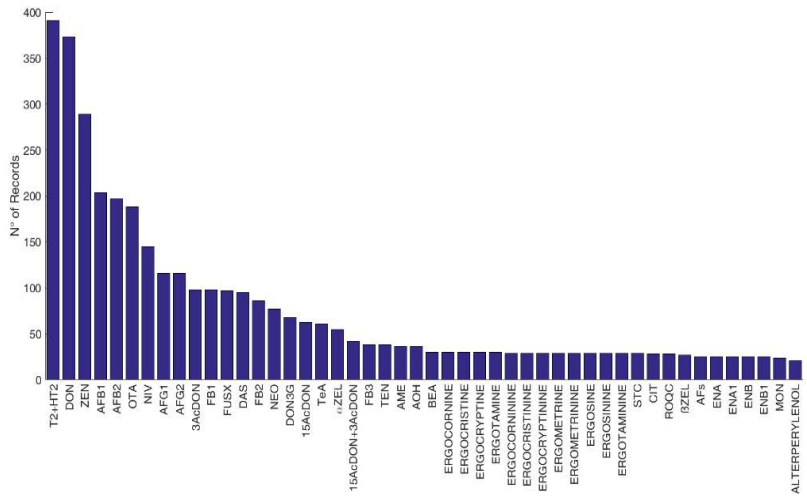

Figure 3. Frequency of reported mycotoxins and secondary metabolites in food in Europe. The figure displays the compounds with a number of records above twenty. $N > 20$: T2: T-2 toxin, HT2: HT-2 toxin, DON: deoxynivalenol, ZEN: zearalenone, AFB1: aflatoxin B1, AFB2: aflatoxin B2, OTA: ochratoxin A, NIV: nivalenol, AFG1: aflatoxin G1,

AFG2: aflatoxin G2, 3Ac-DON: 3-acetyldeoxynivalenol, FB1: fumonisin B1, FUS-X: fusarenon-X, DAS: diacetoxyscirpenol, FB2: fumonisin B2, NEO: neosolaniol, DON3G: deoxynivalenol-3-glucoside, 15Ac-DON: 15acetyldeoxynivalenol, TeA: tenuazonic acid, α-ZEL: α-zearalenol, FB3: fumonisin B3, TEN: tentoxin, AME: alternariol monomethyl ether, AOH: alternariol, BEA: beauvericin, STC: sterigmatocystin, CIT: citrinin, ROQC: Roquefortine C, β-ZEL: β-zearalenol, AFs: total aflatoxins, ENA: enniatin A, ENA1: enniatin A1, ENB: enniatin B, ENB1: enniatin B1, MON: moniliformin. N < 20 (not reported in the figure): FB1+FB2: fumonisin B1 + fumonisin B2, αZEL4G: α-zearalenol-4-glucoside, βZEL4G: β-zearalenol-4-glucoside, T2-triol: T2 triol, ZEN4G: zearalenone-4-glucoside, ZEN4S: zearalenone-4-sulfate, ATX1: altertoxin 1, PAT: patulin, ATX2: Altertoxin 2, AME3G: alternariol monomethyl ether-3-glucoside, AME3S: alternariol monomethyl ether-3-sulfate, AOH3G: alternariol-3-glucoside, AOH3S: alternariol-3-sulfate, FBs: total fumonisins, AOH9G: alternariol-9-glucoside, HFB1: hydrolysed fumonisin B1, FUS: fusaproliferin, MAS: monoacetoxyscirpenol, T2-tetraol: T2 tetraol, ENB4: enniatin B4, STO: scirpentriol, αZEL14G: α-zearalenol-14-glucoside, HT2-3G: HT-2 toxin-3-diglucoside, NIV3G: nivalenol-3-glucoside, β-ZEL14G: β-zearalenol-14-glucoside, ZEN14G: zearalenone-14-glucoside, ZEN14S: zearalenone-14-sulfate, 15OHculmorin: 15-OH Culmorin, 5OHculmorin: 5-OH Culmorin, Culmorin, ENs: enniatins.

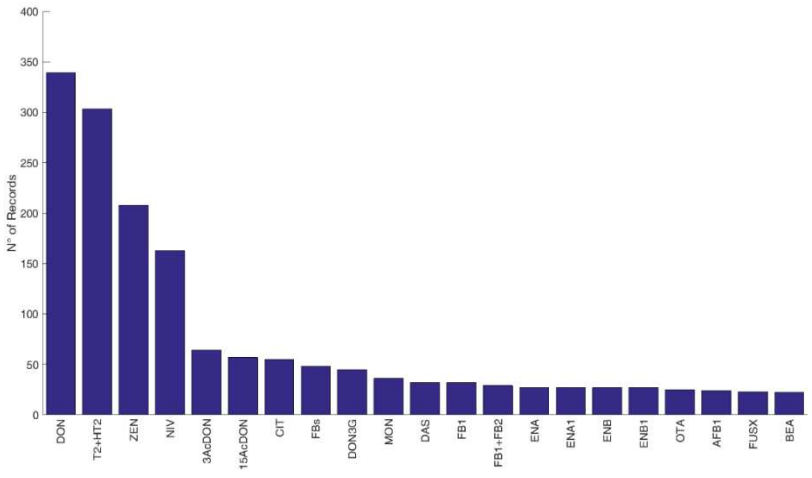

Figure 4. Frequency of reported mycotoxins and secondary metabolites in cereals without specifications of food or feed origin in Europe. The figure displays the compounds with a number of records above twenty. $N > 20$: DON: deoxynivalenol, T2: T-2 toxin, HT2: HT-2 toxin, ZEN: zearalenone, NIV: nivalenol, 3Ac-DON: 3-acetyldeoxynivalenol, 15Ac-DON: 15acetyldeoxynivalenol, CIT: citrinin, FBs: fumonisins, DON3G: deoxynivalenol-3-glucoside, MON: moniliformin, DAS: diacetoxyscirpenol, FB1: fumonisin B1, FB1+FB2: fumonisin B1 + fumonisin B2, ENA: enniatin A, ENA1: enniatin A1, ENB: enniatin B, ENB1: enniatin B1, OTA: ochratoxin A, AFB1: aflatoxin B1, FUS-X: fusarenon-X, BEA: beauvericin. $N < 20$ (not reported in the figure): T2-tetraol: T2 tetraol, FB2: fumonisin B2, NEO: neosolaniol, T2-triol: T2 triol, AME: alternariol monomethyl ether, AOH: alternariol, β-ZEL: β-zearalenol, STO: scirpentriol, 15Ac-DON: 15acetyldeoxynivalenol, α-ZEL: α-zearalenol, AFB2: aflatoxin B2, AFG1: aflatoxin G1, AFG2: aflatoxin G2, FB3: fumonisin B3, Culmorin: culmorin, ENB2: enniatin B2, HFB1: hydrolysed fumonisin B1, OTB: ochratoxin B, ENs: enniatins, Ergometrine/-metrinine, STC: sterigmatocystin, TeA: tenuazonic acid, TEN: tentoxin, 15OHculmorin: 15-OH Culmorin, 2-AOD-3-ol: 2-Amino-14,16-dimethyloctadecan-3-ol, Ergocryptine/-cryptinine, ATX1: altertoxin 1, Aurofusarin, Avenacein Y, Averufin, ENB3: enniatin B3, Equisetin, Ergocristine/-cristinine, ZEN4S:

zearalenone-4-sulfate, Deepoxy HT2, Deepoxy T2, AFs: total aflatoxins, ALT: altenuene.

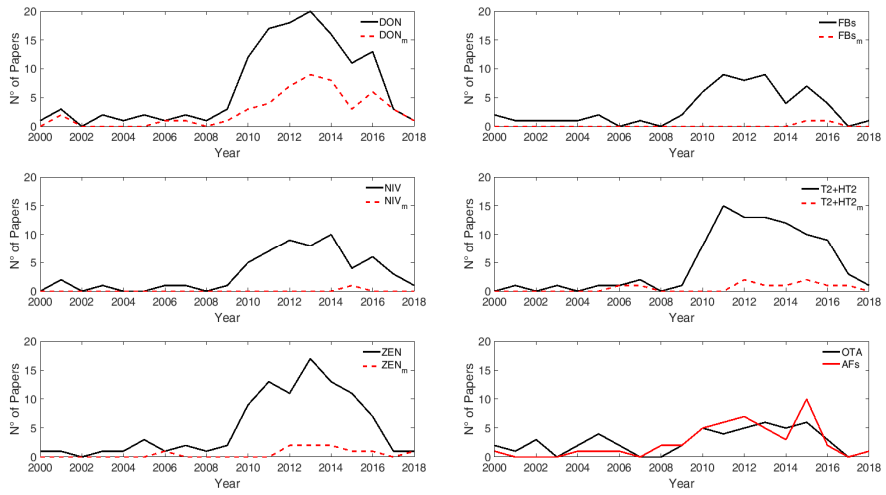

Figure 5. Distribution of records according to year of publication. Solid lines refer to parent mycotoxins and dashed lines refer to modified forms. m = modified forms**.**

3.1. Data Quality

According to the data quality analysis, maize and wheat were the most studied cereals. With regards to wheat, the majority of data was reported for DON which showed the highest score with a value of 4.12/7. In maize, FB_1 showed the highest ranking followed by DON with values of 4.08/7 and 4.06/7, respectively. Overall, DON was among the most reported mycotoxins, ranking first in wheat, barley, cereals, and rye. In maize and oat, DON ranked second after FB_1 and T2+HT2 toxins, respectively. With regards to rice, data were reported mainly on AF and OTA with a general score ranging between 2.89 and 2.77.

Table 2 reports the range obtained for each sub-index forming the total $Score_{gen}$. Figure 6 provides a general view of $Score_{gen}$ index and all sub-indices for combinations of mycotoxin and crops with a score higher than 1.4. After applying quality criteria, a final number of seven crops were selected and used for human exposure assessments to mycotoxins through cereal-based diets.

Table 2. Composition of the $Score_{gen}$ index and range for each individual sub-index

N	Sub-Indices Code	Sub-Indices Meaning	Range	Normalization
1	Score numerosity	data availability	6–332	0–1
2	Score validity	percentage of good data available	0–100	0–1
3	CV score	coefficient of variation of toxin concentration	0–1	0–1
4	P sampleSize	total samples number	1–48	0–1
5	P agePaper	age of papers	2001–2018	0–1
6	P bibIntensity	bibliography intensity	1–215	0–1
7	P haveBounds	statistical information	0–1	0–1

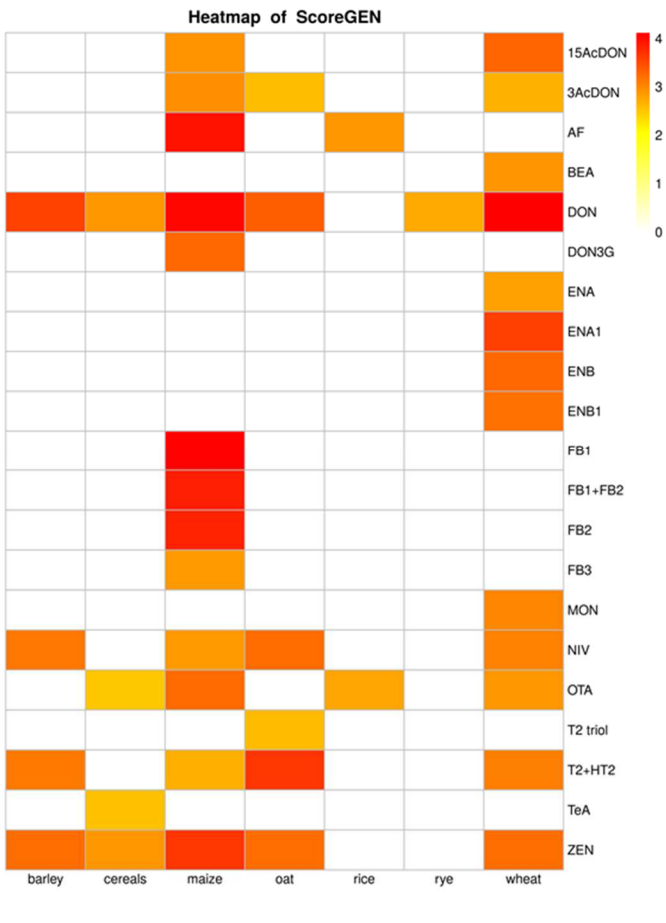

Figure 6. Score$_{gen}$ heatmap for mycotoxin crops with a score higher than 1.4.

3.2. Occurrence of Mycotoxins

LB and UB mean concentrations, as well as maximum concentrations (UB), in food and feed are reported for each crop in the following paragraphs; more details are available in supplementary tables (Tables S1–S6), including concentrations of equivalent mycotoxin (i.e., parent and modified forms) in all cereal-based food categories at a country level in Europe (Table S7). Concentration of equivalent mycotoxins were computed and corrected on the basis of their Potency Factors (PFs) proposed by the EFSA CONTAM Panel (EFSA, 2017a, b, 2018).

3.2.1. Wheat

Wheat was the most reported cereal with regards to individual mycotoxins (34% of total number of records). After maize, wheat contained the highest concentrations of DON reported in food (mean LB–UB: 140.1–187.9 μg/kg) and in feed, mean concentrations were reported as nearly six-fold greater (mean LB–UB: 957.7–1025.4 μg/kg). 15-Ac-DON ranged from mean concentration (LB) of 6.0 μg/kg in food and 139.1 μg/kg in feed; while 3-Ac-DON ranged from mean concentration (LB) of 8.0 μg/kg in food and 11.9 μg/kg in feed. DON3G was reported only in food (mean LB–UB: 18.1–23.6 μg/kg).

The lowest mean concentration of AFB_1 was observed in wheat-based food (mean LB–UB: 0.0–0.6 μg/kg); however, these concentrations increased in feed (mean LB-UB: 7.4–7.6 μg/kg).

Mean concentrations (LB–UB) of ZEN ranged between 24.2–27.0 μg/kg in food and 84.6–85.7 μg/kg in feed; different modified forms were reported, with α-ZEL and β-ZEL as those with the highest mean concentrations.

Wheat was the second cereal with the highest concentration of NIV after oat (mean LB–UB: 54.8–75.2 μg/kg in food; mean LB–UB: 58.2–79.2 μg/kg in feed), and, together with barley, it was the only cereal in which NIV3G was reported.

With regard to feed, the highest concentration of OTA was reported in wheat (mean LB–UB: 12.7–13.4 μg/kg); however, in food the mean concentrations were much lower ranging between 0.5–0.8 μg/kg (LB–UB).

3.2.2. Maize

Maize was the second most reported cereal after wheat with regards to individual mycotoxins and the crop contained the highest mean concentrations of FB_1, both in food (n = 58; mean LB–UB: 540.7–541.3 μg/kg; max: 7878.7 μg/kg) and feed (n = 94; mean LB–UB: 1806.0–1807.1 μg/kg; max: 30,200.0 μg/kg). FB_2 and FB_3 also showed the highest mean concentrations in maize, ranging between 135.6–141.5 μg/kg and 152.6–156.2 μg/kg (LB–UB) in food and 610.7–612.2 μg/kg and 57.5–61.0 μg/kg (LB–UB) in feed, respectively. Overall, FBs were reported mainly individually, and to a lesser extent as the sum of FB_1+FB_2. Scarce data were reported on modified FBs (i.e., hydrolyzed FBs, HFBs) in thermally processed maize (n = 6; FBs+HFBs, mean: 570 μg/kg).

DON was also highly reported in maize both in food (n = 59; mean LB–UB: 256.3–263.2 μg/kg, max: 2266.8 μg/kg) and feed (n = 196; mean LB–UB: 714.9–735.6

μg/kg, max: 9528.0 μg/kg) together with its acetyl derivatives. Mean concentration of 3-Ac-DON and 15-Ac-DON in feed were respectively 26.1–27.1 μg/kg and 87.1–88.1 μg/kg (LB–UB); the lowest concentrations were reported in food for 3-Ac-DON (6.2–6.7 μg/kg), whereas 15-Ac-DON was not reported individually in food, but summed with 3-Ac-DON (mean LB–UB: 186.3–188.6 μg/kg). DON3G was also reported in maize with much higher concentrations in feed (max: 763.0 μg/kg).

AFs were also amongst the most reported mycotoxins, with AFB_1 as the one with the highest mean concentrations (n = 22; mean LB–UB: 1.9–2.2 μg/kg; max: 22.4 μg/kg in food; mean: 9.9 μg/kg; max: 74.8 μg/kg in feed).

Mean concentrations of ZEN ranged between 80.6–82.1 μg/kg (LB–UB) in food and 93.3–94.9 μg/kg (LB–UB) in feed; α-ZEL and β-ZEL were the only modified forms reported in maize.

With regards to T2+HT2, low concentrations were reported in maize compared to other cereals (n = 53; mean LB–UB: 1.8–5.4 μg/kg); higher concentrations were reported in feed compared to food products (n = 174; mean LB–UB: 44.8–49.2 μg/kg). Modified forms were among the most relevant phase I metabolites, namely T2-triol and T2-tetraol, both reported in feed.

Mean concentrations (LB–UB) of NIV ranged between 9.3–28.3 μg/kg in food and 190.6–210.0 μg/kg in feed; no modified forms were reported.

Finally, mean concentrations (LB–UB) of OTA ranged between 0.3–0.6 μg/kg in food and 2.2–2.7 μg/kg in feed.

3.2.3. Barley

Barley was the third most reported cereal with regards to individual mycotoxins after wheat and maize (10% of the total number of records), and showed among the highest mean concentrations for several classes of mycotoxins. With regards to food, barley showed the highest mean concentrations of ZEN (n = 19; mean LB–UB: 26.3–26.4 μg/kg, max: 192.0 μg/kg), OTA (n = 6; mean LB–UB: 1.0–1.1 μg/kg, max: 5.6 μg/kg) and T2+HT2 (n = 48; mean LB–UB: 27.3–30.8 μg/kg, max: 264.0 μg/kg), compared to other crops, and ranked second after maize, rice, and oat, respectively. Barley ranked third with regards to DON in food products (n = 22; mean: 173.8 μg/kg, max: 2029.0 μg/kg); 15-Ac-DON, 3-Ac-DON, and DON3G were also reported. In particular, the highest mean concentrations of DON3G among all cereals were reported in barley in food (n = 5; mean: 109.2 μg/kg, max: 390.0 μg/kg) (when LB–UB is not specified, it meant that the difference between LB and UB concentrations is not perceptible). Whereas, a low number of records was retrieved in feed (n = 3) with a mean DON concentration of 413.7

μg/kg; DON3G was not reported in feed. High mean concentrations were also observed for FB_1 and FB_2, both in food and feed; however, this information was obtained from one single record. Barley reported high concentrations of NIV in food (n = 16; mean LB–UB: 35.2–40.2 μg/kg), ranking third after oat and wheat; NIV3G was reported in one record (25.2 μg/kg). Information on NIV in feed were not retrieved.

3.2.4. Oat

The highest concentrations of NIV were reported in oat, both in food (n = 3; mean LB–UB: 81.4–86.3 μg/kg) and feed (mean LB–UB: 263.3–280.0 μg/kg). FB_1 and FB_2 were reported only in two records respectively, one in food (FB_1: 0.1 μg/kg; FB_2: 0.5 μg/kg) and one in feed (FB_1: 30.0 μg/kg; FB_2: 28.0 μg/kg). DON ranked first among other cereals in feed (n = 6; mean: 1309.7 μg/kg, max: 2690.0 μg/kg), and it was reported also in food with much lower concentrations (n = 31; mean LB–UB: 130.6–132.6 μg/kg, max: 1230.0 μg/kg). Modified forms of DON were also reported; mean concentrations of 3-Ac-DON were higher than 15-Ac-DON both in food (mean LB–UB: 28.5–30.6 μg/kg; mean LB–UB: 6.6–10.8 μg/kg) and feed (mean LB–UB: 127.0–139.5 μg/kg; mean LB–UB: 24.5–49.5 μg/kg). DON3G showed high concentrations in feed (n = 2; mean: 711.0 μg/kg). Scarce information was retrieved on AFs both in food and feed; AFB_1, AFB_2, AFG_1, and AFG_2 were reported in food only in two records, whereas in feed only one record reported AFB_1. It should be noted that the highest concentrations of T2+HT2 were reported in oat both in food (n = 65; mean LB–UB: 179.9–182.5 μg/kg) and feed (n = 17; mean LB–UB: 88.1–96.9 μg/kg).

3.2.5. Rice

The majority of data for individual mycotoxins in rice regarded food commodities where the highest mean concentrations of AFB_1 (n = 124; mean LB–UB: 3.1–3.3 μg/kg; max: 91.7 μg/kg) and OTA (n = 44; mean: 2 μg/kg in food) were reported. Low mean concentrations of FB_1 (n = 3; mean LB–UB: 0.0–8.4 μg/kg; max: 12.5 μg/kg), FB2 (n = 1; mean LB–UB: 0.0–0.5 μg/kg; max: 0.5 μg/kg), DON (n = 22; mean LB–UB: 7.9–15.6 μg/kg; max: 96.0 μg/kg), T2+HT2 (n = 14; mean LB–UB: 0.0–8.9 μg/kg; max: 60.0 μg/kg), and ZEN (n = 7; mean LB–UB: 0.0–6.6 μg/kg; max: 10.1 μg/kg) were reported. No information was retrieved on modified forms in rice except for 3-Ac-DON reported in four records with mean ranging (LB–UB) between 0.0 and 0.6 μg/kg. Five records were also reported on NIV (mean LB–UB:

0.0–16.0 μg/kg; max 75.0 μg/kg). In feed, only two mycotoxins were reported, namely DON and T2+HT2.

3.2.6. Rye

Overall, scarce information was available on rye compared to other cereals; the number of records ranged between one and 18, and the majority of the data retrieved was for food commodities. It could be emphasized that rye showed the highest mean concentration of OTA (mean LB–UB: 0.8–0.9 μg/kg). However, this information was derived from a limited number of records (n = 5). DON was reported both in food (n = 11; mean LB–UB: 55.9–56.8 μg/kg) and feed (n = 2; mean: 56.2 μg/kg). Whereas 15-Ac-DON (n = 2; mean LB–UB: 0.5–3.0 μg/kg) and 3-Ac-DON (n = 5; mean LB–UB: 8.6–13.6 μg/kg) were reported only in food.

3.3. Co-Occurrence of Mycotoxins

The main co-occurring mycotoxins were analyzed by crop category. The analysis of the data quality led to the identification of five suitable crop categories, namely maize, wheat, oat, barley, and cereals. The latter was often reported even if the composition and/or the percentages of ingredients were not always indicated by the authors. However, considering that the consumption of mixed cereal grains-based commodities is also one of the causes of the natural co-occurrence of mycotoxins both in animal and human diets, this information was kept.

Several surveys reported the natural co-occurrence of mycotoxins, and most of them concerned DON, OTA, NIV, ZEN, and T2+HT2. Less data were found for AFs, ENs, and *Alternaria* toxins.

For each crop aggregation and co-occurrence, average concentrations were then calculated (Figure 7) In detail, for each paper reporting on co-occurrence for barley, maize, oat, and wheat, the concentration of each co-occurring mycotoxin is reported as the mean value.

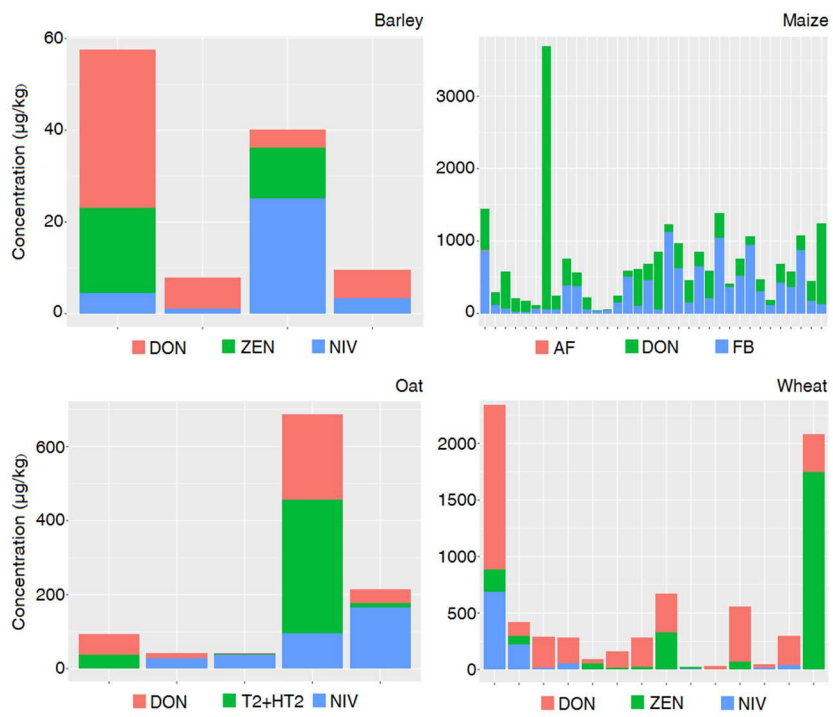

Figure 7. Concentrations of each co-occurring mycotoxin for barley, maize, oat, and wheat.

3.4. Results of Multinomial Analysis

The multinomial analysis provided a simulation model that allowed prediction of potential co-occurrence patterns for two or more mycotoxins based on the observed patterns reported in the literature. Probabilities of mycotoxin co-occurrence for one or more mycotoxins were simulated for records above the LOD and are reported below. Figure 8 shows the number and type of observed patterns of co-occurrence of native mycotoxins in barley, maize, oat, and wheat, while the probabilities simulated by the multinomial model are reported in Table 3. In maize, DON and FB have the highest probability of co-occurrence (74.4%), whereas the probability of DON, FB, and AF is rather low (1.0%). In barley and wheat, the combination of DON and ZEN is the most probable; whereas DON and T2+HT2 have the highest simulated probability of co-occurring in oat.

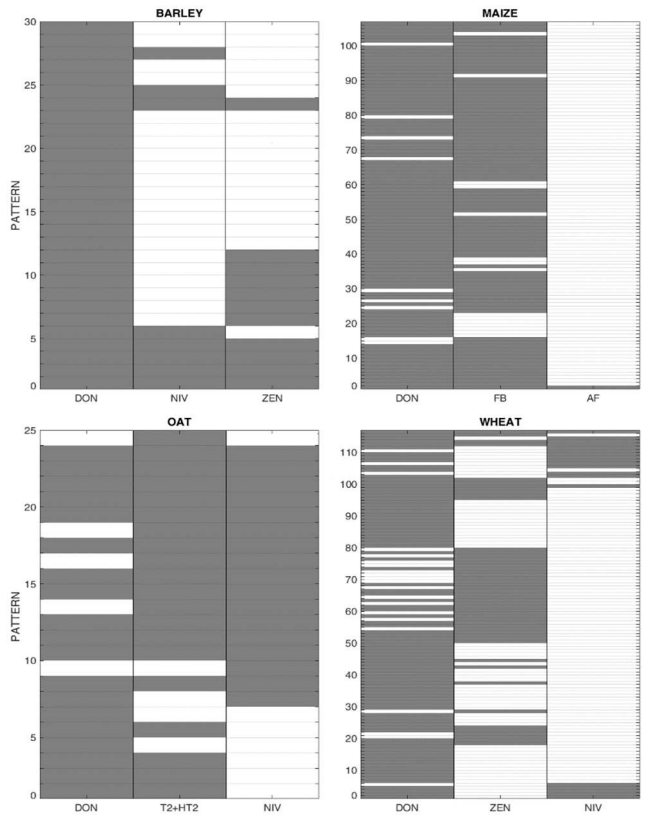

Figure 8. Total number of observations with specific patterns of mycotoxin co-occurrence. Grey and white boxes display the presence and absence of mycotoxins, respectively.

Table 3. Probability simulated by the multinomial model of having co-occurring mycotoxins in maize, barley, oat, and wheat.

Pattern	DON	NIV	ZEN	%	Pattern	DON	FB	AF	%

		Barley					**Maize**		
1			1	1.3	1		1		10.7
2		1		0.8	2		1	1	0.5
3		1	1	4.5	3	1			13.1
4	1			20.5	4	1		1	0.3
5	1		1	32.9	5	1	1		74.4
6	1	1		25.8	6	1	1	1	1.0
7	1	1	1	14.2					
Pattern	**DON**	**T2/HT2**	**NIV**	**%**	**Pattern**	**DON**	**NIV**	**ZEN**	**%**
		Oat					**Wheat**		
1			1	3.0	1			1	2.7
2		1		5.0	2		1		0.2
3		1	1	22.3	3		1	1	5.0
4	1			3.0	4	1			18.1
5	1		1	18.8	5	1		1	46.1
6	1	1		25.4	6	1	1		15.0
7	1	1	1	22.5	7	1	1	1	12.9

4. DISCUSSION

Cereals are often contaminated with a wide range of mycotoxins and other fungal metabolites. Unsurprisingly, wheat and maize were the most reported cereals with the highest concentrations of FBs, DON, AFs, and ZEN.

FBs were widely reported in maize foods and feed for which the maximum concentrations of FB_1+FB_2 exceeded the legal maximum levels (MLs) of 1000 and 4000 μg/kg, respectively (European Commission, 2007).

In the context of food, the max concentrations of DON in barley, maize, oat, and wheat exceeded the legal limits of 750 μg/kg (European Commission, 2006b, 2007); however, when looking at mean concentrations, none of the cereals showed very high concentrations. Similar results were observed in feed except that max concentrations in barley did not exceed the MLs of 1250 μg/kg in contrast to maize, oat, and wheat (European Commission, 2006b, 2007).

In line with pre-existing knowledge, maximum concentrations of T2+HT2 were particularly high in oat and oat-containing foods, exceeding the MLs of 200 μg/kg (European Commission, 2013).

AFs were predominantly reported in rice and maize as a result of a pre- and post-harvest colonization of the grains with *A. flavus* (Gonçalves et al., 2019a). In addition, in rice, high concentration of OTA was also reported in food, exceeding the legal limits of 3.0 μg/kg (European Commission, 2006b). These results are in agreement with the well-known rice contamination with the OTA-producer *Aspergillus ochraceus*.

Contamination with NIV was more relevant for oat, wheat, and barley, however, MLs have not been set in the current regulation for either NIV nor for its metabolites (European Commission, 2006b).

With regards to occurrence of native forms, DON, FBs, and ZEN showed the highest simulated potential co-occurrence value, and in particular, DON was more probable to be found in co-occurrence with FBs in maize and with ZEN in wheat. This finding is consistent with the results of a recent study conducted on Canadian cereal samples where the co-occurrence of DON and other *Fusarium* mycotoxins was frequently observed in wheat and barley (Shi et al., 2019).

Overall, the data collection exercise concludes that occurrence of modified forms are mostly reported in food compared to feed. Apart from the occurrence of ZEN and its phase I and phase II modified forms, only a limited number of quantitative data are available for other modified forms; i.e., acetyl derivatives of DON, hydrolyzed FBs, phase I metabolites of T2, and NIV3G. In addition, data are still scarcely and unevenly reported regardless of an increased awareness of the

contribution of modified forms to the toxicity of mycotoxins. Liquid chromatography (LC) coupled with mass spectrometry (MS) has only recently become widely used for the determination of multiple mycotoxins which partly explain why literature data are still scarce on the co-occurrence of modified forms (Malachová et al., 2018). In general, promising progresses have been recently observed in the context of analytical methods, providing a positive indication of forthcoming improvements for the simultaneous determination of multiple mycotoxins, both of different native toxins and modified forms. Yet, analytical methods are still a limiting factor for a complete data collection, both for the cost and the lack of suitable protocols.

In summary, the large body of evidence collected in this study highlights that wheat and maize may contribute significantly to mycotoxin co-exposure in human and animal species compared to other crops. The results indicate that mycotoxin co-occurrence is common in European cereal-based feed and food, and further highlights the need to conduct monitoring studies for multiple mycotoxins. Such studies would also support filling considerable data gaps regarding the co-occurrence of mycotoxins and their modified forms. Further research efforts are needed to identify co-occurrence patterns of multiple mycotoxins in the real world and these will allow provision of a scientific basis to understand the combined toxicity of mycotoxins, the relative contribution of the parent compounds compared to metabolites, and modified forms and their likely interactions.

5. CONCLUSIONS

Cereals and related processed food products are frequently contaminated with mycotoxins, and co-occurrence of *Fusarium* mycotoxins is highly reported in cereals of major consumption in human and animal species, particularly wheat, maize, barley, and oat. However, 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. Therefore, the challenge of depicting realistic patterns of co-exposure in humans and animals remains. To bring forward the risk assessment of mycotoxin mixture, the refinement of assessment factors to determine safe levels of exposure is needed, and the following is recommended:

(1) The necessity of continuous monitoring of the major mycotoxins in different agricultural commodities and the creation of harmonized methods for generating accurate (co-)occurrence data is strongly suggested. This is mandatory to provide consistent and coherent data for mycotoxin co-occurrence and will

allow risk modelling to prioritize key congeners of human and animal health relevance;

(2) LODs and LOQs for mycotoxins and the analytical method used may vary significantly across studies and across measurements. It is known that the degree of LCD in the dataset has a large impact on the uncertainty of the exposure assessment; this uncertainty is further magnified when assessing exposure to multiple chemical substances. Thus, a more harmonized approach should be adopted to reduce this source of uncertainty but also to allow the usability of published data that, currently, in some cases are unusable (e.g., authors reporting a range of LOD/LOQ across different classes of mycotoxins);

(3) More accurate reporting of geographical information of the samples could also optimize the efforts to better understand and map the mycotoxin problem in the EU.

In this context, this article provides a source of ready-to-use data for the implementation of exposure assessments of multiple mycotoxins in food and feed.

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Table S1. Occurrence and co-occurrence of DON and secondary metabolites (µg/kg) for Barley, Cereals, Maize, Oat, Rice, Rye and Wheat for feed and food products.

			FOOD					FEED				
			15AcDON	**15+3AcDON**	**3AcDON**	**DON**	**DON3G**	**15AcDON**	**15+3AcDON**	**3AcDON**	**DON**	**DON3G**
Barley	**N**		5	4	6	22	5	1		1	3	
	Mean Conc	**LB**	19.6	0.3	22.3	173.8	109.2	0.0		0.0	413.7	
	Conc	**UB**	21.6	1.0	26.7	173.8	109.2	50.0		50.0	413.7	
	Max Conc	**UB**	97.0	1.0	120.0	2029.0	390.0	50.0		50.0	600.0	
Cereals	**N**		17		24	21	6				2	
	Mean Conc	**LB**	9.3		14.3	46.9	22.8				543.0	
	Conc	**UB**	13.1		17.4	50.1	24.2				543.0	
	Max Conc	**UB**	119.0		130.0	132.1	29.0				884.0	
Maize	**N**			15	5	59	15	51		51	196	72
	Mean Conc	**LB**		186.3	6.2	256.3	0.0	87.1		26.1	714.9	112.1
	Conc	**UB**		188.6	6.7	263.2	5.3	88.1		27.1	735.6	117.0
	Max Conc	**UB**		808.1	31.0	2266.8	5.3	1047.0		339.0	9528.0	763.0
Oat	**N**		21		24	31	6	2		4	6	2
	Mean Conc	**LB**	6.6		28.5	130.6	34.2	24.5		127.0	1309.7	711.0
	Conc	**UB**	10.8		30.6	132.6	36.8	49.5		139.5	1309.7	711.0
	Max Conc	**UB**	27.0		116.0	1230.0	97.0	50.0		341.0	2690.0	806.0
Rice	**N**				4	22					1	
	Mean Conc	**LB**			0.0	7.9					800.0	
	Conc	**UB**			0.6	15.6					800.0	

	Max Conc	**UB**			0.6	96.0					800.0	
Rye	**N**		2		5	11					2	
	Mean Conc	**LB**	0.5		8.6	55.9					56.2	
		UB	3.0		13.6	56.8					56.2	
	Max Conc	**UB**	5.0		43.2	277.0					83.1	
Wheat	**N**		16	23	22	162	33	19		19	41	
	Mean Conc	**LB**	6.0	2.8	8.0	140.1	18.1	139.1		11.9	957.7	
		UB	55.9	7.5	14.6	187.9	23.6	142.6		16.4	1025.4	
	Max Conc	**UB**	150.0	64.8	59.0	1657.0	250.0	1575.0		93.8	12270.0	

LB: lower-bound scenario where the concentration of non-detected analyte is zero and the concentration of detected but non-quantified analyte is the limit of detection. UB: upper-bound scenario where the concentration of non-detected analyte is the limit of detection and the concentration of detected but non-quantified analyte is the limit of quantification. Max Conc refers to maximum upper bound concentration value. Blanck cells refer to not available information. N: number of records.

Table S2. Occurrence and co-occurrence of FB and secondary metabolites (μg/kg) for Barley, Cereals, Maize, Oat, Rice, Rye and Wheat for feed and food products.

			FOOD						**FEED**					
			FB_1	**FB_1+FB_2**	**FB_2**	**FB_3**	**FBs**	**FBs+HFBs**	**FB_1**	**FB_1+FB_2**	**FB_1+FB_2+FB_3**	**FB_2**	**FB_3**	**FBs**
Barley	**N**		1	1	1		1		1			1		
	Mean Conc	**LB**	156.3	0.0	65.0		0.0		0.0			0.0		
		UB	156.3	100.0	65.0		25.0		30.0			30.0		
	Max Conc	**UB**	156.3	100.0	65.0		25.0		30.0			30.0		

Cereals	N		5	1	4	1								
	Mean	LB	8.9	0.0	19.3	0.0								
	Conc	UB	9.9	100.0	20.5	5.0								
	Max Conc	UB	35.0	100.0	75.0	5.0								
Maize	N		58	13	54	23	7	6	94	13	5	85	45	37
	Mean	LB	540.7	823.8	135.6	152.6	472.8	570.0	1806.0	2611.8	7220.0	610.7	57.5	681.8
	Conc	UB	541.3	823.8	141.5	156.2	473.7	570.0	1807.1	2611.8	7220.0	612.2	61.0	795.8
	Max Conc	UB	7878.7	4092.0	1563.6	1066.1	1300.5	1651.0	30200.0	7890.0	11100.0	13200.0	246.0	5727.0
Oat	N		1	1	1				1			1		
	Mean	LB	0.0	0.0	0.0				0.0			28.0		
	Conc	UB	0.1	100.0	0.5				30.0			28.0		
	Max Conc	UB												
Rice	N		3		1									
	Mean	LB	0.0		0.0									
	Conc	UB	8.4		0.5									
	Max Conc	UB	12.5		0.5									
Rye	N			1			1							
	Mean	LB		0.0			6.2							
	Conc	UB		100.0			6.2							
	Max Conc	UB		100.0			6.2							
Wheat	N		17	1	17	14	1		17			17	16	2
	Mean	LB	8.1	0.0	2.5	0.0	0.0		4.6			1.6	0.0	551.25
	Conc	UB	10.1	100.0	3.8	0.7	25.0		13.8			9.4	7.7	667.25

	Max Conc	**UB**	131.2	100.0	35.9	6.0	25.0		78.0			30.0	8.4	1102.5

LB: lower-bound scenario where the concentration of non-detected analyte is zero and the concentration of detected but non-quantified analyte is the limit of detection. UB: upper-bound scenario where the concentration of non-detected analyte is the limit of detection and the concentration of detected but non-quantified analyte is the limit of quantification. Max Conc refers to maximum upper bound concentration value.

Table S3. Occurrence and co-occurrence of AF and secondary metabolites (μg/kg) for Barley, Cereals, Maize, Oat, Rice, Rye and Wheat for feed and food products.

			FOOD					**FEED**				
			AFB_1	**AFB_2**	**AFG_1**	**AFG_2**	**AFs**	**AFB_1**	**AFB_2**	**AFG_1**	**AFG_2**	**AFs**
Barley	**N**		3	1	1	1	5	1				
	Mean Conc	**LB**	0.2	0	0.1	0	0	0				
		UB	0.2	0	0.1	0	0.4	0.2				
	Max Conc	**UB**	0.4	0	0.1	0	1.8	0.2				
Cereals	**N**		13	14	14	13	1					
	Mean Conc	**LB**	0	0	0	0	0					
		UB	0.6	0.9	1.1	0.2	0.4					
	Max Conc	**UB**	3	10	5	0.4	0.4					
Maize	**N**		22	22	22	22	3	35	6	6	6	27
	Mean Conc	**LB**	1.9	0.1	0	0	3.6	9.9	1.3	2.8	1.1	4.2
		UB	2.2	0.8	0.4	0.3	3.7	9.9	1.3	2.8	1.1	5.5
	Max Conc	**UB**	22.4	10	5	1	10.3	74.8	3.2	14	3.2	67
Oat	**N**		2	2	2	2	2	1				
	Mean Conc	**LB**	0	0.8	0	0	0	0				

		UB	1.6	0.9	2.8	0.2	0.4	0.2				
	Max Conc	**UB**	3	1.6	5	0.3	0.4	0.2				
Rice	**N**		124	120	35	35	5					
	Mean Conc	**LB**	3.1	0.2	10.7	7.8	1.4					
		UB	3.3	0.5	10.9	7.8	1.5					
	Max Conc	**UB**	91.7	12.1	78.7	31	1.9					
Rye	**N**		1	1	1	1	2					
	Mean Conc	**LB**	0	0	0	0	0					
		UB	0.9	0.2	2.2	0.4	1.1					
	Max Conc	**UB**	0.9	0.2	2.2	0.4	1.8					
Wheat	**N**		34	33	33	33	4	24	9	9	9	
	Mean Conc	**LB**	0	0	0.2	0	0.7	7.4	0	0	0	
		UB	0.6	0.1	1.1	0.3	0.9	7.6	0.2	0.2	0.2	
	Max Conc	**UB**	3	0.2	6.6	0.9	2.6	143.6	0.3	0.3	0.3	

LB: lower-bound scenario where the concentration of non-detected analyte is zero and the concentration of detected but non-quantified analyte is the limit of detection. UB: upper-bound scenario where the concentration of non-detected analyte is the limit of detection and the concentration of detected but non-quantified analyte is the limit of quantification. Max Conc refers to maximum upper bound concentration value.

Table S4 a. Occurrence and co-occurrence of ZEN and secondary metabolites (µg/kg) for Barley, Cereals, Maize, Oat, Rice, Rye and Wheat for food products.

			FOOD										
			αZEL	**αZEL14G**	**αZEL4G**	**ßZEL**	**ßZEL14G**	**ßZEL4G**	**ZEN**	**ZEN14G**	**ZEN14S**	**ZEN16G**	**ZEN4G**	**ZEN4S**
Barley	**N**		3	1		3	1		19	1	1	1		

	Mean Conc	LB	0.2	2.9		0.7	0.7		26.3	2.7	10.6	0.3		
		UB	9.9	2.9		11.7	0.7		26.4	2.7	10.6	0.9		
	Max Conc	UB	27.0	2.9		31.0	0.7		192.0	2.7	10.6	0.9		
Cereals	N		6		5	6		5	18				5	6
	Mean Conc	LB	32.7		0.0	24.7		0.0	10.6				9.6	4
		UB	34.7		9.0	28.5		9.0	11.5				14.4	13.2
	Max Conc	UB	110.0		9.0	86.0		9.0	53.0				20.0	24
Maize	N		15						37					
	Mean Conc	LB	0.0						80.6					
		UB	2.5						82.1					
	Max Conc	UB	2.5						823.0					
Oat	N		8		7	8		7	26				7	6
	Mean Conc	LB	16.1		0.0	19.5		2.9	11.4				3.4	2.0
		UB	19.5		9.0	24.1		10.6	13.0				11.4	11.2
	Max Conc	UB	68.0		9.0	96.0		20.0	85.0				16.0	12.0
Rice	N								7					
	Mean Conc	LB							0					
		UB							6.6					
	Max Conc	UB							10.1					
Rye	N		1			1			7					

	Mean Conc	**LB**	0.0			0.0			7.0					
		UB	2.0			2.0			7.3					
	Max Conc	**UB**	2.0			2.0			41.0					
Wheat	**N**		22	1	6	9	1	6	165	1	1	1	6	6
	Mean Conc	**LB**	3.2	3.1	0.0	18.2	0.0	0.0	24.2	0.6	4.9	2.1	2.7	3.7
		UB	5.7	3.1	9.0	22.3	0.2	9.0	27.0	0.6	4.9	2.1	10.7	11.0
	Max Conc	**UB**	39.0	3.1	9.0	104.0	0.2	9.0	856.0	0.6	4.9	2.1	16.0	11.0

LB: lower-bound scenario where the concentration of non-detected analyte is zero and the concentration of detected but non-quantified analyte is the limit of detection. UB: upper-bound scenario where the concentration of non-detected analyte is the limit of detection and the concentration of detected but non-quantified analyte is the limit of quantification. Max Conc refers to maximum upper bound concentration value.

Table S4 b. Occurrence and co-occurrence of ZEN and secondary metabolites (μg/kg) for Barley, Cereals, Maize, Oat, Rice, Rye and Wheat for feed products.

			FEED											
			αZEL	**αZEL14G**	**αZEL4G**	**ßZEL**	**ßZEL14G**	**ßZEL4G**	**ZEN**	**ZEN14G**	**ZEN14S**	**ZEN16G**	**ZEN4G**	**ZEN4S**
Barley	**N**								3					
	Mean Conc	**LB**							16.3					
		UB							16.3					
	Max Conc	**UB**							27.0					
Cereals	**N**								5					

	Mean Conc	**LB**							79.9						
		UB							79.9						
	Max Conc	**UB**							134.0						
Maize	**N**		2			2			122						
	Mean Conc	**LB**	9.0			91.5			93.3						
		UB	9.0			91.5			94.9						
	Max Conc	**UB**	15.0			166.0			2180.0						
Oat	**N**		2	1		2	1		5	1	1	1			
	Mean Conc	**LB**	69.0	0.0		1.5	0.0		44.2	0.1	31.6	4.2			
		UB	69.0	0.5		17.0	0.2		44.2	0.3	31.6	4.2			
	Max Conc	**UB**	136.0	0.5		31.0	0.2		77.0	0.3	31.6	4.2			
Rice	**N**														
	Mean Conc	**LB**													
		UB													
	Max Conc	**UB**													
Rye	**N**														
	Mean Conc	**LB**													
		UB													
	Max Conc	**UB**													
Wheat	**N**		7						24						
	Mean Conc	**LB**	3.5						84.6						
		UB	4.1						85.7						

	Max Conc	**UB**	10.0						555.0					

LB: lower-bound scenario where the concentration of non-detected analyte is zero and the concentration of detected but non-quantified analyte is the limit of detection. UB: upper-bound scenario where the concentration of non-detected analyte is the limit of detection and the concentration of detected but non-quantified analyte is the limit of quantification. Max Conc refers to maximum upper bound concentration value.

Table S5. Occurrence and co-occurrence of T2-HT2 and secondary metabolites (µg/kg) for Barley, Cereals, Maize, Oat, Rice, Rye and Wheat for feed and food products.

			FOOD						**FEED**					
			T2 tetraol	**T2 triol**	**T2 G**	**T2+HT2**	**HT23 G**	**HT2 G**	**T2 tetraol**	**T2 triol**	**T2 G**	**T2+HT2**	**HT23 G**	**HT2 G**
Barley	**N**		2	2		48	1				18	45		18
	Mean Conc	**LB**	51.4	10.3		27.3	3.6				2.4	53.3		48.2
		UB	51.4	10.3		30.8	10.8				2.4	55.6		48.2
	Max Conc	**UB**	102.7	20.4		264.0	10.8				14.5	213		162.8
Cereals	**N**					58						13		
	Mean Conc	**LB**				2.8						27.7		
		UB				9.7						27.8		
	Max Conc	**UB**				60.0						65.1		
Maize	**N**					53			3	2		174		
	Mean Conc	**LB**				1.8			117.7	42		44.8		
		UB				5.4			117.7	42		49.2		

	Max Conc	**UB**				60.0			301	76		2300		
Oat	**N**		1	13		65			1	1		17	1	
	Mean Conc	**LB**	3.6	20.3		179.9			150	19		88.1	41.4	
		UB	3.6	21.9		182.5			150	19		96.9	41.4	
	Max Conc	**UB**	3.6	122		2570.0			150	19		196	41.4	
Rice	**N**					14						1		
	Mean Conc	**LB**				0.0						76		
		UB				8.9						76		
	Max Conc	**UB**				60.0						76		
Rye	**N**		1	1		18								
	Mean Conc	**LB**	1.8	0		11.9								
		UB	1.8	1		15.0								
	Max Conc	**UB**	1.8	1		90.0								
Wheat	**N**		2	2		116	1		1			41		
	Mean Conc	**LB**	4.8	0.3		7.7	15		38			15.6		
		UB	4.8	0.8		15.8	15		38			21.9		
	Max Conc	**UB**	9.2	1		123.0	15		38			135		

LB: lower-bound scenario where the concentration of non-detected analyte is zero and the concentration of detected but non-quantified analyte is the limit of detection. UB: upper-bound scenario where the concentration of non-detected analyte is the limit of detection and the concentration of detected but non-quantified analyte is the limit of quantification. Max Conc refers to maximum upper bound concentration value.

Table S6. Occurrence and co-occurrence of NIV, NIV3G and OTA (µg/kg) for Barley, Cereals, Maize, Oat, Rice, Rye and Wheat for feed and food products.

			FOOD			FEED		
			NIV	**NIV3G**	**OTA**	**NIV**	**NIV3G**	**OTA**
Barley	**N**		16	1	6			5
	Mean Conc	**LB**	35.2	25.2	1.0			10.0
		UB	40.2	25.2	1.1			12.0
	Max Conc	**UB**	180.0	25.2	5.6			25.7
Cereals	**N**		16		22			
	Mean Conc	**LB**	3.3		0.4			
		UB	5.5		0.4			
	Max Conc	**UB**	35.8		2.2			
Maize	**N**		21		32	89		68
	Mean Conc	**LB**	9.3		0.3	190.6		2.2
		UB	28.3		0.6	210.0		2.7
	Max Conc	**UB**	175.7		4.8	2547.0		51.0
Oat	**N**		20		4	3	1	1
	Mean Conc	**LB**	81.4		0.1	263.3	36.9	0.0
		UB	86.3		0.5	280.0	36.9	10.0
	Max Conc	**UB**	208.0		1.0	635.0	36.9	10.0
Rice	**N**		5		44			
	Mean Conc	**LB**	0.0		2.0			
		UB	16.0		2.0			
	Max Conc	**UB**	75.0		27.3			
Rye	**N**		9		5			4
	Mean Conc	**LB**	12.0		0.8			6.5
		UB	14.6		0.9			6.5

	Max Conc	**UB**	56.9		2.1			14.5
Wheat	**N**		47	1	50	19		24
	Mean Conc	**LB**	54.8	23.1	0.5	58.2		12.7
		UB	75.2	23.1	0.8	79.2		13.4
	Max Conc	**UB**	302.4	23.1	3.9	690.0		267.0

LB: lower-bound scenario where the concentration of non-detected analyte is zero and the concentration of detected but non-quantified analyte is the limit of detection. UB: upper-bound scenario where the concentration of non-detected analyte is the limit of detection and the concentration of detected but non-quantified analyte is the limit of quantification. Max Conc refers to maximum upper bound concentration value.

Chapter III - Human and animal risk assessment of mycotoxin mixture in maize using the component-based approach

Roberta Palumbo [1], Jean-Lou Dorne [2], and Paola Battilani [1]

[1]Faculty of Agriculture, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29100 Piacenza, Italy
[2]European Food Safety Authority (EFSA), Via Carlo Magno 1A, 43126, Parma, Italy

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INTRODUCTION

The harmonised framework for risk assessment of combined exposure to multiple chemicals follows the well-defined steps of risk assessment. First, the question to be addressed is identified (**problem formulation**), then exposure is quantified (**exposure assessment**) and toxicological effects are identified and characterised to determine safe levels of exposure (**hazard identification and characterization**). The exposure and hazard metrics are finally compared so that the risk to human and animal health can be characterised (**risk characterisation**) (EFSA, 2019a). The two common approaches that are applied to human and animal health risk assessment of combined exposure to multiple chemicals are 'whole mixture' and 'component-based' approaches. The choice is based on the degree of characterisation of a mixture, being the whole mixture approach relevant for mixture whose composition is only partially known and 'component-based approach' used for chemically well-characterised mixture. Thus, if the individual components of the mixture and their exposure levels are chemically defined, the component-based approach (CBA) is recommended. Over the years, this approach has been applied to a number of case studies dealing with several chemicals, mainly pesticides or food additives (EFSA, 2008a; WHO, 2009; EFSA, 2013c, b).

The framework for risk assessment of combined exposure to multiple chemicals using the CBA applies a tiered system. Extrapolation of existing information from the real data for the prediction of health risks of combined exposure to chemicals requires methods to reduce complexity of the system and constrain focus on key questions while allowing flexibility of the assessment process. One recommended way to assess mixtures is the use of tiers of increasing complexity (EFSA, 2008b; Solomon et al., 2008; EFSA, 2019a). The tiering principle was firstly introduced in the context of ecotoxicology and then applied to human and animal health risk assessment. It is based on the amount of accurate and representative information available, and as one ascends through the tiers (i.e. 0, 1, 2, 3), the estimates of exposure become more realistic. Likewise, the lower the number of accessible data, the lower the number of the tier.

According to the framework, the components of the mixture are organised into chemical assessment groups (AGs) by applying a common risk assessment principle (i.e. the grouping criteria). For each AG, quantitative predictions of combined toxicity are derived from knowledge of the toxicity of the individual components and the default assumption of dose addition, unless evidence is available. This implies that every toxicant contributes to the combination effect in proportion to its dose and individual potency.

The present study aims to investigate the applicability of harmonised methodologies based on the EFSA guidance document to mycotoxins mixture in maize through a scenario of possible co-exposure in humans and animals. Thus, a human and animal risk assessment to mycotoxin mixture in maize was conducted using a modelled component-based approach for selected mixture of mycotoxins that, according to our data, co-occur in maize based feed and food products. Mycotoxin occurrence data in maize-based feed and food were collected from the Literature and EFSA database, and were used to estimate potential pattern of co-exposure in humans and animals. Available hazard information for each mycotoxin of the assessment group was collected. Reference points (RPs) (i.e. No-Observed-Adverse-Effect-Level (NOAEL) and benchmark dose lower confidence limit (BDML)) were extracted from EFSA opinions and chemical hazard database (OpenFoodTox). An important consideration in applying component-based approaches is whether and how to account for potential interactions between components. Interactions are defined as joint action between multiple chemicals that differ in dose addition or response addition categorised as less than additive (antagonism, inhibition, masking) or greater than additive (synergism, potentiation). It must be noted that changes in toxicokinetic (TK) aspects and interaction effects of mycotoxins have been associated with multi-mycotoxin co-exposure. Therefore, TK aspects after concurrent exposure to mycotoxins in living organism were collected for further refinement of hazard characterization (Gkrillas et al. *accepted*). However, in the Literature there are still limited papers addressing TK aspects (*in vitro* and *in vivo*) to multiple mycotoxins co-exposure in comparison with exposure of the single compounds, and, in absence of evidence dose addition was adopted as a conservative default assumption.

The observed patterns of co-exposure as well as hazard assessment were used for organizing mycotoxins into assessment groups (AGs). For risk characterisation, dose addition was applied using a margin of exposure approach (MOE). The MOE is defined by EFSA as 'the reference point on the dose-response curve divided by the estimated intake in humans' (EFSA, 2005a). The MOE approach uses a reference point corresponding to a dose that cause a low but measurable response in animals. This RP is compared with dietary exposure estimates; a small MOE represents a higher risk than a larger MOE. For substances that are not genotoxic a 100-fold uncertainty factor is usually applied to allow for species differences and human variability (WHO, 2009). Thus, MoE superior to 100 is interpreted as a scenario of low concern whereas a MOE inferior to 100 suggests the need to refine the risk assessment or that the compounds in the assessment group may be of concern (EFSA, 2005a). Additional uncertainties are included for genotoxic and carcinogenic substances (i.e. inter-individual variability in the carcinogenic

process), MoE of 10,000 or higher, are interpreted as a scenario of low concern (Renwick, 1999).

1. METHODS

1.1. Collection of mycotoxin occurrence data in maize-based feed and food

For the purpose of human and animal risk assessment of multiple mycotoxins in maize-based diet, (co)occurrence data in maize-based feed and food was collected from two sources: (i) data extracted from the Literature (Palumbo, *et al*., submitted) and (ii) data from the EFSA Chemical Occurrence Database. These two databases were analysed separately as described in the following paragraphs. However, the same methodology was applied to both databases in order to allow a comparison between the two different sources of information.

1.1.1. Occurrence data from the Literature

An Extensive Literature Search (ELS) was undertaken in order to collect available papers in scientific literature on the (co-)occurrence of mycotoxins in maize in EU countries from 2010-2018. Nonetheless, the paucity of relevant data made searching the scientific literature published up to 2000 necessary to reach a more robust data set for modelling. The derived database on maize (from now on referred as Literature database) comprises an initial total number of 2,215 analytical results encompassing fourteen classes of main mycotoxins (Table 1). To ensure appropriate quality of the data, a 7-scaled quality index was applied. For more details on methodology in data collection, extraction and quality assessment please refer to (Palumbo, *et al*., submitted). The analytical results are stored either at aggregated level or at sample level. In particular, Literature database accounts only for records reporting the mean concentration of total number of samples (MeanTot), the mean concentration of positive samples (MeanPos) or concentrations at sample level. In absence of these values, when the minimum and maximum value analysed and the percentage of samplings above the LOD was reported, the median values were considered. Since the collection of these data was meant to estimate maize dietary exposure in humans and animals, data were stratified by feed and food commodities. Information on co-occurrence of mycotoxins was stored when clearly stated by the author (for aggregated results) or when the analytical results were reported at sample level. The frequency in which a mycotoxin was reported alone or in combination with others was recorded, allowing the identification of patterns of co-occurrence and their frequency in the dataset studied.

Table 1: List of mycotoxins extracted from Literature and EFSA database

Mycotoxin		Literature	EFSA	References
Class	**Forms and abbreviations**			
Aflatoxins	Aflatoxin B1 (AFB1), AFB2, AFG1, AFG2	1	1	(EFSA, 2019b)
Alternaria toxins	alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), altenuene (ALT)	2	-	(EFSA, 2011a)
Beauvericin	BEA	3	-	(EFSA, 2014c)
Citrinin	CIT	4	2	(EFSA, 2012c)
Diacetoxyscirpenol	4,15-Diacetoxyscirpenol (DAS)	5	-	(EFSA, 2018d)
Deoxynivalenol and its metabolites	Deoxynivalenol (DON), 3-acetildeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), deoxynivalenol-3-glucoside (DON3G)	6	3	(EFSA, 2017c)
Enniatins	Enniatin A (ENA), ENA1, ENB, ENB1	7	4	(EFSA, 2014b)
Ergot alkaloids	Ergocornine, Ergocristine, Ergocryptine, Ergometrine, Ergonovine, Ergosine, Ergotamine and the corresponding -inine epimers	8		(EFSA, 2012b)

Fumonisins	Fumonisin B1 (FB1), FB2, FB3	9	5	(EFSA, 2018c)
Nivalenol	NIV	10	6	(EFSA, 2013d)
Ochratoxins	Ochratoxin A (OTA)	11	7	(EFSA, 2015b)
Sterigmatocystin	STC	12	8	(EFSA, 2015b)
T-2 toxin, HT-2 toxin	T2, HT2	13	9	(EFSA, 2017a)
Zearalenone and its metabolites	Zearalenone (ZEN), Zearalanone (ZAN), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL)	14	10	(EFSA, 2017b)

1.1.2. Occurrence data from EFSA database

An initial number of 23,754 analytical results on mycotoxins in maize were extracted from the EFSA Chemical Occurrence Database. Data stored in this database comes from European national authorities and similar bodies, research institutions, academia, food business operators and other stakeholders[1]. The most important classes of mycotoxins and their most common modified forms have been extracted according to last EFSA opinions (Table 1). To ensure an appropriate quality of the data used in the exposure assessment, the initial extracted data set was evaluated applying exclusion criteria. Special attention was paid to different parameters such as:

[1] https://www.efsa.europa.eu/en/microstrategy/contaminants-occurrence-data

- Year of sampling. The data collection before 2010 does not guarantee the quality of the data required for this analysis, thus only the data sampled from 2010 onwards were retained for the assessment;
- Analytical methods. Only analytical methods reliable for determination of the mentioned mycotoxins were included;
- Codification of samples under FoodEx classification (EFSA, 2011c). Analytical results reported as grains with not defined end-use (i.e. 'Corn grain as crop') were excluded;
- LOD/LOQ cut-off identified by the CONTAM Panel in the recent EFSA opinions were applied per mycotoxin and per analytical method, as reported in Appendix A (Table A.1)

After applying the exclusion criteria, a final number of 21,551 analytical results (analysed from 6,914 samples) were included in the final dataset. Data were stratified in two blocks, namely feed (named as 'Maize' in FoodEx classification, n=10735) and food (named as 'Corn grain' in FoodEx classification, n=10816). Occurrence data for total aflatoxin (AFs) was generated by summing up the analytical results of the individual aflatoxins (i.e. AFB1, AFB2, AFG1 and AFG2). The same approach was used for calculating the occurrence data for total fumonisins (FBs) (i.e. FB1, FB2 and FB3), DON and its metabolites (i.e. 15-AcDON, 3-AcDON and DON3G) as well as ZEN and its metabolites (i.e. ZAN, α-ZAL, β-ZAL, α-ZEL and β-ZEL).

The frequency in which a mycotoxin was reported alone or in combination with others was recorded, allowing the identification of patterns of co-occurrence and their frequency in the dataset studied. This will be used to fit a multinomial model in order to estimate the probability that each of the mycotoxin is present in a sample from food or feed. The multinomial model uses the frequencies of each combination of mycotoxin to estimate the probability that a single mycotoxin is present, and this is then used to simulate potential co-occurrence based on the observed patterns reported.

1.1.3. Building lower bound and upper bound scenarios for occurrence of mycotoxins

In both datasets, the left censored data (results below LOD (non-detected analytes) or below LOQ (detected but non-quantified analytes)) were treated by the substitution method as recommended in the 'Principles and Methods for the Risk

Assessment of Chemicals in Food' (WHO/IPCS, 2009). The same method is indicated in the EFSA scientific report 'Management of left-censored data in dietary exposure assessment of chemical substances' (EFSA, 2010). This guidance suggests that the lower bound (LB) and upper bound (UB) approach should be used for chemicals likely to be present in the food (e.g. naturally occurring contaminants, nutrients and mycotoxins). At the LB, all results reported as lower than the LOD have been set to zero or to the numerical value of LOD for results reported as lower than LOQ; at the UB, the results below the LOD have been set to numerical value of LOD and to the value of LOQ for results below the LOQ. This means that the uncertainty associated to the exposure estimations due to censored data was taken into consideration.

LOD/LOQ values vary across measurements in relation to the mycotoxin and the analytical method used, as shown in Appendix A (Table A.1). Therefore, in absence of unique values of the LOD/LOQ per mycotoxin and to account for such uncertainty, two approaches have been used to build the LB/UB scenarios: (i) the mean and (ii) the highest value of LOQ/LOD among the different analytical method for each class of mycotoxin (Table 2). These two approaches are from now on referred as 'max LOD/LOQ' and 'mean LOD/LOQ'.

Table 2: Max and mean LOD and LOQ values used to estimate the LB and UB scenarios

Mycotoxin	Max (µg/kg)		Mean (µg/kg)	
	LOD	**LOQ**	**LOD**	**LOQ**
AF	10	44	1.3	6.1
FB	300	1000	233.3	683.3
DON	28	380	24.7	148.5
OTA	0.6	2	0.4	0.8
T2+HT2	5	100	3	10
ZEN	10	200	10	76.7

1.1.4. Distribution fitting

Occurrence data were used to determine the best fitting distribution in order to build a reliable reference distribution useful for each mycotoxin for each dataset (i.e. feed or food and each class of mycotoxin at aggregate level (sum of native form and its modified forms)). The resulting distributions allow simulating model to be built that mimics the potential co-occurrence of several mycotoxins based on

the observed patterns reported in the Literature and in EFSA. The fitted distributions provide the basis of a simulated model to derive potential exposure for animals and humans based on occurrence in maize. The distributions considered were Weibull, Gamma, Exponential, Log Normal and Normal and, to select the best fitting distribution, the Akaike information criterion (AIC) has been used (Akaike, 1973). The distribution with the smallest AIC was then selected as the best fitting distribution for each mycotoxin. Maximum likelihood method was used to estimate the parameters of the distributions for each mycotoxin based on their occurrence values, using the function rriskMLEdist from the package rriskDistributions in R (Belgorodski et al., 2017).

1.2. Consumption data

1.2.1. Feed consumption data

Currently, comprehensive feed consumption databases reporting amounts or types of feed consumed by livestock in the EU are not available. Consequently feed intake values are based on estimates of feed intakes published by the EFSA panel on additives and products or substances used in animal Feed (FEEDAP) (EFSA et al., 2017) (Table 3).

Table 3: Default values for daily feed intake scaled to body weight for pigs and chicken

Animal category	**Default values daily feed intake (g DM/kg body weight)**	**Body weight (kg)**	**Feed intake (kg per day)**	**Ref**
Chicken for fattening	79	2	0.158	(EFSA et al., 2017)
Laying hen	53	2	0.106	
Piglet	44	20	0.88	
Pig for fattening	37	60	2.20	

1.2.2. Food consumption data

Consumption data in humans have been gathered from the EFSA Comprehensive European Food Consumption Database (hereinafter referred as Comprehensive Database) which provides a compilation of existing national information in EU on food consumption at the individual levels (EFSA, 2011c). The latest version of the Comprehensive Database, updated in 2018, reports results from a total of 60 different dietary surveys carried out in 25 different Member States covering 119,458 individuals. For chronic exposure assessment to maize products, food consumption data were available from 31 different dietary surveys carried out in 17 different European countries.

Within these dietary studies, subjects are classified in different age classes. For this case study three classes have been considered, namely 'Adolescents' (≥ 10 years to < 18 years old), 'Adults (≥ 18 years to < 65 years old) and 'Elderly' (≥ 65 years to < 75 years old).

In the Comprehensive Database consumption data are classified according to FoodEx2 classification system. FoodEx2 is a food classification system that simplifies the linkage between occurrence and food consumption data. The system consists of a large number of individual food items aggregated into food categories which are further divided into subgroups in a hierarchical fashion with seven levels (EFSA, 2015a). In order to estimate the chronic dietary exposure for maize products, consumption data for average consumers have been extracted for maize commodities at FoodEx2 Level 4 (i.e. maize and similar, maize semolina and maize starch). Here, the average consumption for the three maize-food commodities was calculated and used for exposure to avoid a too conservative approach.

Table 4: Consumption EFSA Foodex2-level-4 (maize products) chronic consumers only (g/kg bw per day)

Age class	Foodex2-level-4	Mean	Ref
Adolescents	Maize and similar	0.134	EFSA
Adolescents	Maize semolina	0.002	Comprehensive
Adolescents	Maize starch	0.017	Database
Adults	Maize and similar	0.127	
Adults	Maize semolina	0.263	
Adults	Maize starch	0.018	
Elderly	Maize and similar	0.054	
Elderly	Maize semolina	0.292	

Elderly	Maize starch	0.027

1.3. Exposure assessment

Exposure assessment was performed as the product between occurrence data and consumption data for humans and animals (Section 2.2). A graphical stepwise approach of exposure assessment using the component-based approach is given below (
Figure 2).
Based on co-occurrence patterns (section 2.1) and hazard information (section 2.3), the multiple mycotoxins were grouped into assessment groups. Thus, chronic exposure estimates (EXP) were calculated for each individual mycotoxin using equation 1. For farm animals, exposures were derived by combining estimated feed intakes (Table 3) with the occurrence of individual mycotoxins (mean and P95) in maize feed samples at LB/UB (Eq. 1). The following species were covered: (i) poultry (fattening chicken and laying hen) and (ii) pigs (piglet and fattening pigs).

$$EXP_i\ (\mu g/kg\ bw\ per\ day) = \frac{Feed\ intake\ (kg\ per\ day)\ *\ Concentration\ of\ mycotoxin\ (\mu g/kg)}{kg\ bw}$$

(Equation 1)

where i is an index indicating the mycotoxin (AF, etc.)

With regards to humans, the exposures were estimated for average consumers (average consumption in three subpopulations) using the LBUB in µg/kg bw per day for each mycotoxin (Section 2.2). Occurrence data and consumption data were linked at the relevant FoodEx level (i.e. L4: maize and similar, maize semolina, maize starch) (Eq. 2).

$$EXP_i\ (\mu g/kg\ bw\ per\ day) = \left(\frac{Consumption\ (g/day/kg\ bw)}{1000}\right) \times Concentration\ of\ mycotoxin\ (\mu g/kg)$$

(Equation 2)

where i is an index indicating the mycotoxin (AF, etc.)

The exposure assessment started from a low tier in the light of: (i) type of assessment required (aggregate exposure assessment (feed and food)), (ii) occurrence and consumption data for either the specific raw commodities to be

considered and (iii) hazard data (deterministic values with limited mechanistic information).

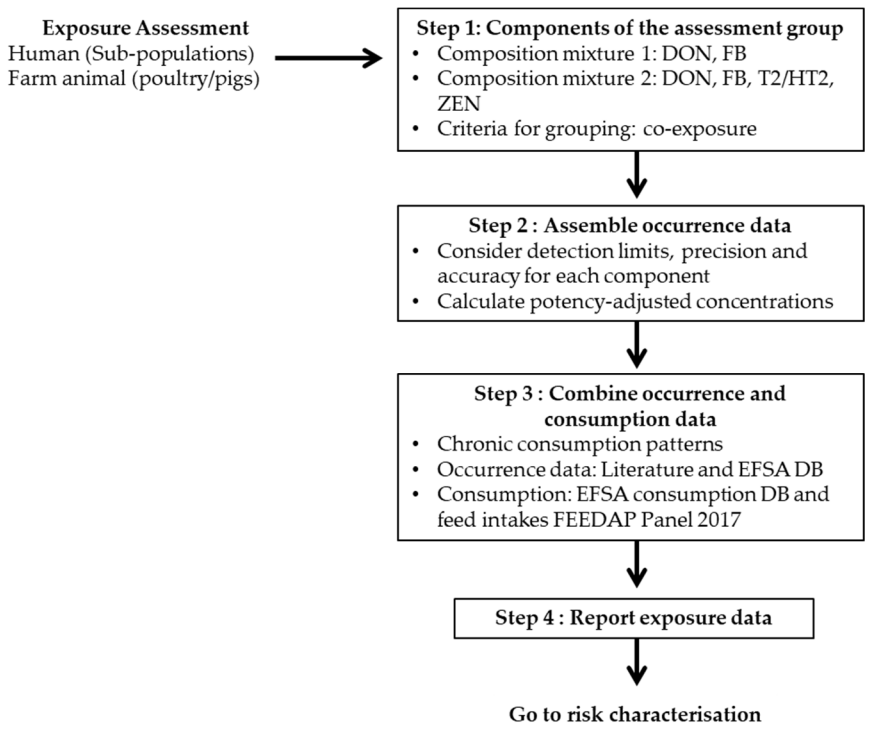


Figure 2: Step wise approach of exposure assessment using the component-based approach from (EFSA, 2019a)

1.4. Hazard identification and characterisation: selection of reference points

Toxicity studies are designed to identify the adverse effects produced by a substance and to characterise the dose–response relationships for the adverse effects detected. The data obtained in these studies are used to derive a dose that may be of relevance for human and animal health, the so-called reference point (RP) or point of departure (PoD). Traditionally, the NOAEL has been used as the RP for estimating the health-based guidance values (HBGVs) in risk assessment of non-genotoxic substances. In 2005, the use of the benchmark dose (BMD) approach was introduced for deriving the RP for substances that are both genotoxic and carcinogenic (EFSA, 2005a; JECFA, 2006), and, recently, this approach has been

confirmed also for non-genotoxic substances (EFSA, 2017d). The benchmark dose is a 'standardised reference point derived from the animal data by mathematical modelling within the observed range of experimental data' (EFSA, 2017d). The Scientific Committee recommends the use of the $BMDL_{10}$ (benchmark dose lower confidence limit 10%) which is an estimate of the lowest dose which is 95% certain to cause no more than a 10% cancer incidence in rodents. In our studies, the RPs were extracted from EFSA opinions and the chemical hazard database called OpenFoodTox (Section 2.3) (Dorne et al., 2017). An equivalent factor approach is proposed using simply the RPs as conservative estimates. The equivalent factors (EFs) were calculated using the most potent compound for which the EF is considered to be equal to 1 and calculating the EFi of each mycotoxin following the equation below (Eq. 3).

$$EF_i = \frac{RP_{Most\ Potent\ Mycotoxin}}{RP_i} \qquad \text{(Equation 3)}$$

where i is an index indicating the mycotoxin (AF, etc.)

A graphical step wise approach (Figure 3) is given below:

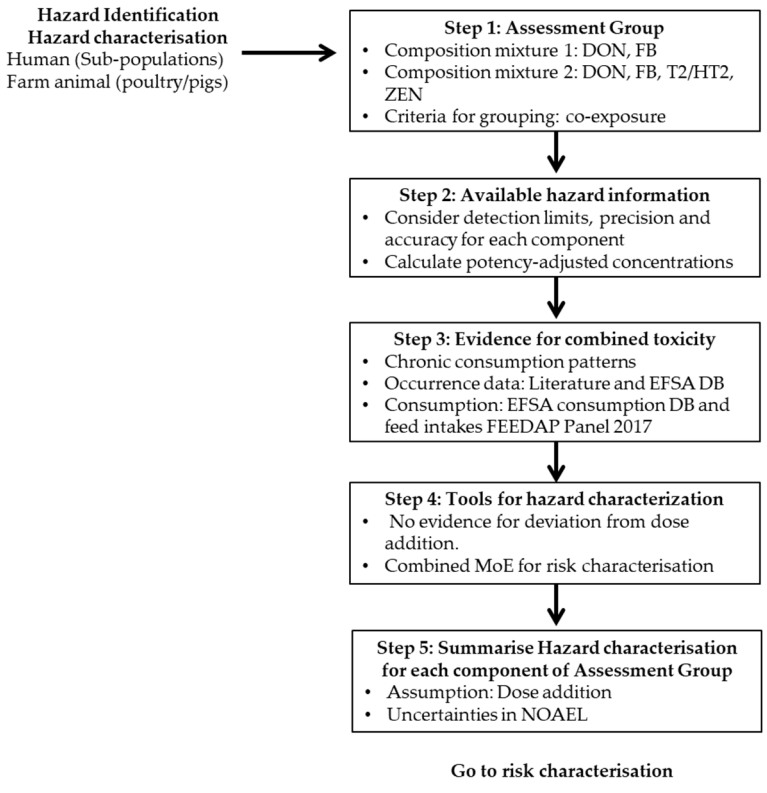


Figure 3: Step wise approach of hazard assessment using the component-based approach from (EFSA, 2019a)

1.5. Risk characterisation

For risk characterisation, dose addition is applied using a margin of exposure approach (MOE). Exposure metrics to each individual mycotoxins of the mixture were corrected using the EF, and the total exposure (exposure to the mixture - Expmix) has been calculated by multiplying the exposure metrics of each single compound by its related EF and by summing all of them, so that:

$$Exp_{mix} = (Exp_i \times EF_i) + \cdots + (Exp_i \times EF_i) \quad \text{(Equation 4)}$$

where i is an index indicating the mycotoxin (AF, etc.)

From the total exposure, a MOE of the mixture was estimated as the ratio between the RP of the most potent compound and the sum of normalised individual exposures Sum(Expi)*(EFi) (eq. 5).

$$MOE_{mix} = \frac{RP_{Most\ Potent\ Mycotoxin}}{EXP_{mix}}$$

(Equation 5)

To account for inter and intraspecies variability and the uncertainty that they introduce in the risk assessment of chemicals, an uncertainty factor of 100-fold is usually applied (Dorne et al., 2005). MOE superior to a 100-fold was interpreted as a scenario of low concern for compounds that are not genotoxic and carcinogenic whereas an MOE inferior to 100-fold suggests the need to refine the risk assessment or that the compounds in the assessment group may be of concern. For compounds that are genotoxic and carcinogenic, a MOE superior to a 10,000-fold is interpreted as of low concern and MOEs inferior to such a value are suggest either the need to refine the risk assessment or a concern for risk management (EFSA, 2005a).

A graphical step wise approach (Figure 4) is given below:

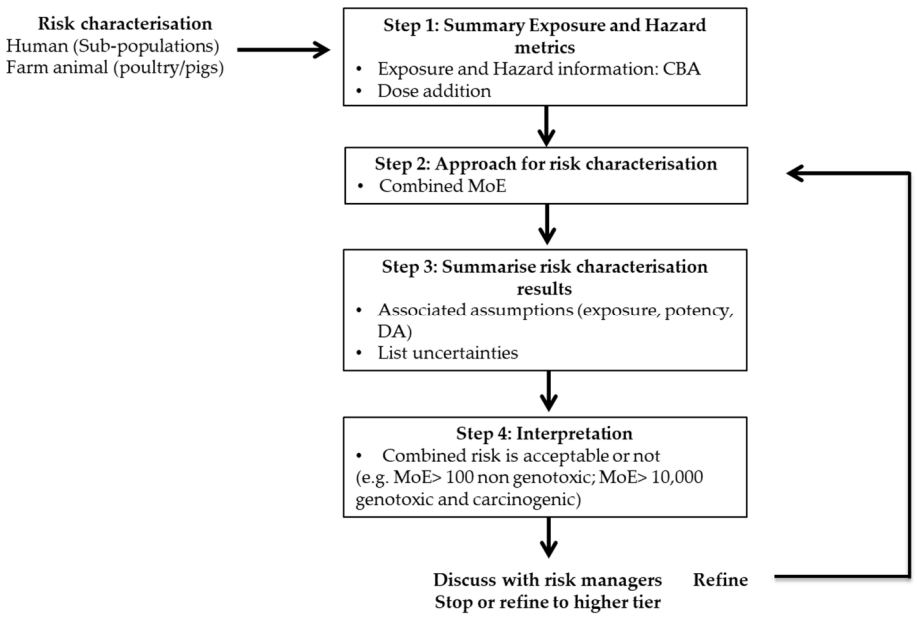


Figure 4: Step wise approach of risk assessment using the component-based approach from (EFSA, 2019a)

2. RESULTS

2.1. Occurrence data

2.1.1. Occurrence data from Literature

After applying quality criteria, a final number of 1,789 analytical results (analysed from 11,624 samples) were included in the final Literature database (n=1,261 for feed and n=528 for food) encompassing eight (8) classes of mycotoxin (i.e. AF, CIT, DON, EN, FB, NIV, OTA, T2/HT2 and ZEN). The samples were collected between 2000 and 2018 and the number of samples per year is presented in
Figure 5. Figure 6 shows the distribution of analytical results for each class of mycotoxin by European countries. The major contributing countries were Spain and Italy, followed by Portugal and Austria. However, it should be noted that the origin of samples was not always the European country where the study was performed, i.e. the data set also contained a limited number of samples originating from South America, Africa and Asia (n= 41 analytical results).

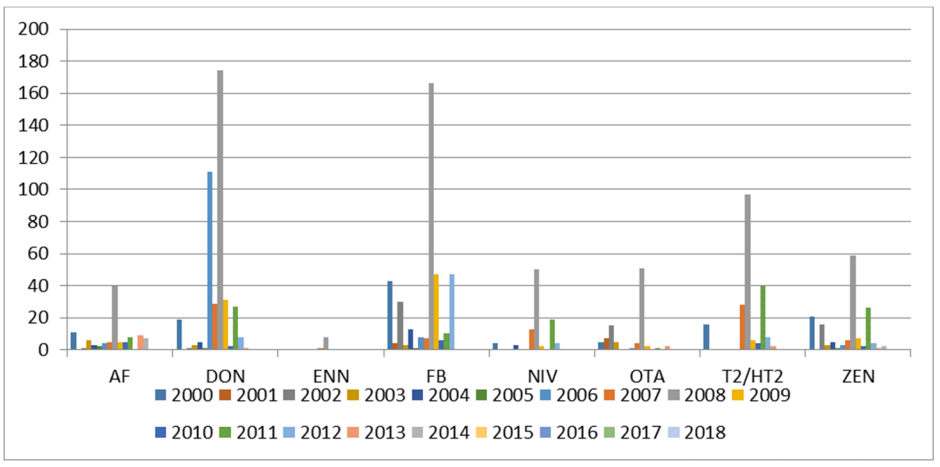
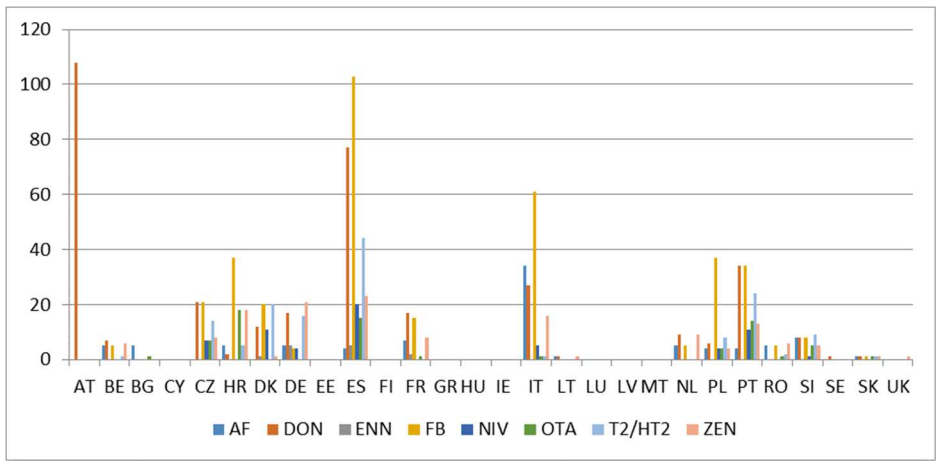


Figure 5: Distribution of analytical results for AFs, DONs, ENNs, FBs, NIV, OTA, T2/HT2 and ZEN by sampling year



AT, Austria; BE, Belgium; BG, Bulgaria; CY, Cyprus; CZ, the Czech Republic; DE, Germany; DK, Denmark; EE,Estonia; ES, Spain; FI, Finland; FR, France; GR, Greece; HR, Croatia; HU, Hungary; IE, Ireland; IT, Italy; LT, Lithuania; LU, Luxembourg; LV, Latvia; MT, Malta; NL, the Netherlands; NO, Norway; PL, Poland; PT, Portugal; RO, Romania; SE, Sweden; SI, Slovenia; SK, Slovakia; UK, the United Kingdom

Figure 6: Distribution of analytical results for AFs, DONs, ENNs, FBs, NIV, OTA, T2/HT2 and ZEN by European countries

The percentage of analytical results reported at aggregated level and at sample level are 31% and 69%, respectively. Distribution of analytical results per class of mycotoxin and percentage of left-censored data (LCD) (i.e. below LOD and LOQ) are reported in Table 5.

The data set comprises a total of 263, 58, 53 and 87 analytical results respectively on DON, 3-Ac-DON, 15-Ac-DON and DON3G. Analytical results on the two acetylated forms of DON were also reported as the sum of two (n=17). The proportion of LCD ranged from 18% for DON to about 80% for its modified forms. After DON, the most frequently reported mycotoxins were total FBs (n=461), being FB1 the most reported with a percentage of 10% of LCD. A total of 166 analytical results from 4151 samples were available for ZEN. Modified forms of ZEN (phase I metabolites) were also reported, namely α-ZEL and β-ZEL (n=23). The proportions of left-censored data were about 4% for ZEN, and 85% for its modified forms.

The data set comprises 180 results on total aflatoxins (AFs), (i.e. AFB1, AFB2, AFG1 and AFG2), being AFB1 the most representative across aflatoxins (i.e. 60 analytical results from 2778 samples). Analytical results were reported either as individual analytical results for AFB1, AFB2, AFG1 and AFG2 and as the sum of four (n=36). Occurrence data for the sum of AFs concentrations were obtained by summing the

available individual concentrations of the individual AFs for each sample or analytical results and subsequently combining them with data reported as sum of the four.
T2/HT2 toxins and OTA accounted for 239 and 111 analytical results, respectively. Whereas, fewer analytical results were available on ENs, i.e. 4 analytical results from 169 samples collected from four papers published between 2010 and 2016. Analytical results were reported mainly as individual ENs (i.e. ENA, ENA1, ENB and ENB) but in one case also as the sum of the four. The proportion of LCD ranged from about 30% and 70%.
Not all analytical results of Literature database were considered for dietary exposure; mycotoxins with limited number of analytical results and/or high percentage of LCD results were excluded in the present assessment.

Table 5: Distribution of analytical results per mycotoxin across feed and food

Mycotoxin	N	N samples	LCD %
AFs			
Aflatoxin B1	60	2778	17%
Aflatoxin B2	28	1068	11%
Aflatoxin G1	28	1068	15%
Aflatoxin G2	28	1068	18%
AFB1+AFB2+AFG1+AFG2	36	588	38%
DONs			
DON	263	777	18%
3-AcDON	58	319	87%
15-AcDON	53	201	56%
3AcDON+15AcDON	15	15	40%
DON3G	87	391	85%
ENs			
ENA	1	70	73%
ENA1	1	70	30%
ENB	1	169	53%
ENB1	1	70	30%
FBs			
FB1	164	2318	10%
FB2	143	1489	18%
FB3	69	235	16%
NIV	113	776	70%
OTA	111	2293	35%
T2/HT2	239	2042	37%
ZEN			
ZEN	166	4151	4%

α-ZEL and β-ZEL	23	149	85%

N: number of analytical results; % LCD: proportion of left-censored data

2.1.2. *Occurrence data from EFSA*

A total number of 21,551 analytical results (analysed from 6,914 samples) were included in the final EFSA dataset. The samples were collected between 2010 and 2018 and the number of samples per year is presented in Figure 7. Figure 8 shows the distribution of analytical results for each class of mycotoxin by European countries. The analytical results included in the final data set were collected in 24 different European countries, and the major contributing countries were Bulgaria, France and Germany. However, it should be noted that the origin of samples was not always the reporting European country, i.e. the data set also contained samples originating from South America, Africa and Asia (n= 1698 analytical results).

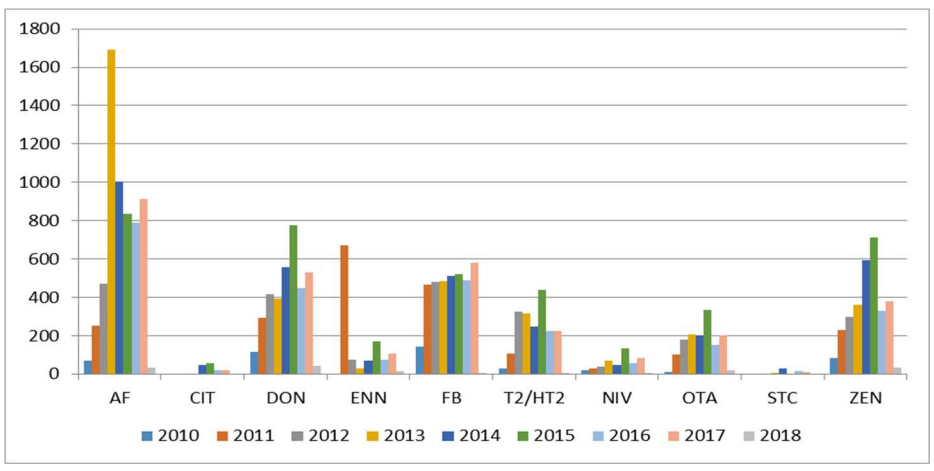
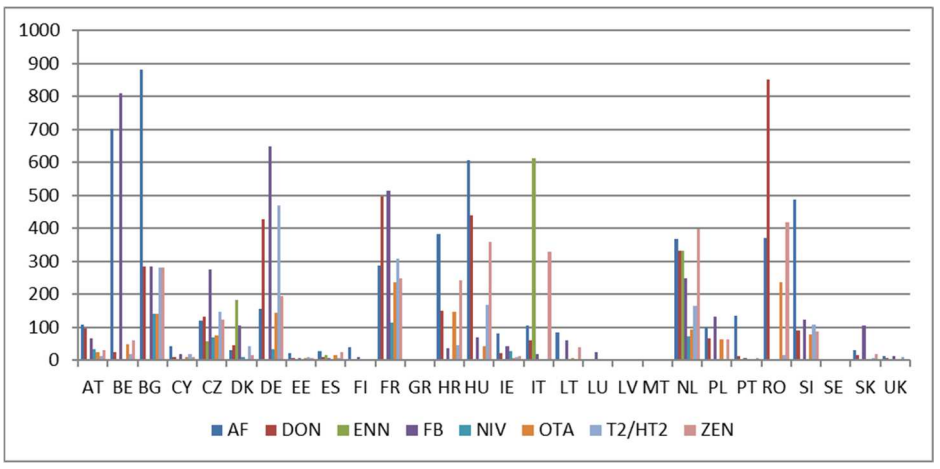


Figure 7: Distribution of analytical results for AFs, CIT, DONs, ENNs, FBs, T2/HT2, NIV, OTA, STC and ZEN by sampling year



AT, Austria; BE, Belgium; BG, Bulgaria; CY, Cyprus; CZ, the Czech Republic; DE, Germany; DK, Denmark; EE,Estonia; ES, Spain; FI, Finland; FR, France; GR, Greece; HR, Croatia; HU, Hungary; IE, Ireland; IT, Italy; LT, Lithuania; LU, Luxembourg; LV, Latvia; MT, Malta; NL, the Netherlands; NO, Norway; PO, Poland; PT, Portugal; RO, Romania; SE, Sweden; SI, Slovenia; SK, Slovakia; UK, the United Kingdom

Figure 8: Distribution of analytical results for AFs, CIT, DONs, ENNs, FBs, NIV, OTA, STC, T2/HT2 and ZEN by European countries

The data set comprises 6,054 results on total aflatoxins (AFs), (i.e. AFB1, AFB2, AFG1 and AFG2), being AFB1 the most representative across aflatoxins (n=3,390). After AFs, the most frequently reported mycotoxins were total FBs (n=3,681), DON (n=2,648), ZEN (n=2,486), T2/HT2 toxins (n=1,927) and OTA (n=1,426). Whereas a very restricted number of analytical results were available on NIV (n=485), CIT (n=141) and STC (n=58). Table 6 shows the number of analytical results and the percentage of LCD per mycotoxins.

Overall, 76% of the analytical results were reported as below the LOD or LOQ, accounting for 88%, 55%, 63%, 92%, 84% and 69% respectively for AFs, DON, FBs, OTA, T2/HT2 and ZEN. High proportion of LCD (92%) was observed in ENs (n=1200).

The limited number of analytical results for certain class of mycotoxins (i.e. CIT, NIV and STC) made impossible to fit any distribution and therefore they were excluded from risk assessment. ENs were also excluded due to the high proportion of LCD that would have resulted in high uncertainty.

Table 6: Distribution of analytical results per mycotoxin across feed and food

Mycotoxin	N	% LCD
AFs	6054	88%
Aflatoxin B1	3390	82%
Aflatoxin B2	889	96%
Aflatoxin G1	889	95%
Aflatoxin G2	886	98%
CIT	141	90%
DONs	3565	55%
DON	2648	48%
3-AcDON	511	90%
15-AcDON	406	61%
ENs	1200	92%
ENA	300	97%
ENA1	300	96%
ENB	300	86%
ENB1	300	91%
FBs	3681	63%
FB1	1653	46%
FB2	1614	75%
FB3	414	89%
NIV	485	79%
OTA	1426	92%
T2/HT2	1927	84%
T2 toxin	1006	86%
HT2 toxin	921	83%
STC	58	97%
ZENs	3014	69%
ZEN	2486	63%
ZAN	136	84%
α-ZEL	84	99%
β-ZEL	84	100%
β-ZAL	224	100%

N: number of analytical results; % LCD: proportion of left-censored data

2.1.3. *Distribution fitting*

2.1.3.1. *Literature data*

Figure 9 and Figure 10 show two examples of plot on data fitting distribution concerning total AFs in feed and food, respectively. Data and fitting distributions for other mycotoxins are shown in Appendix B.1.

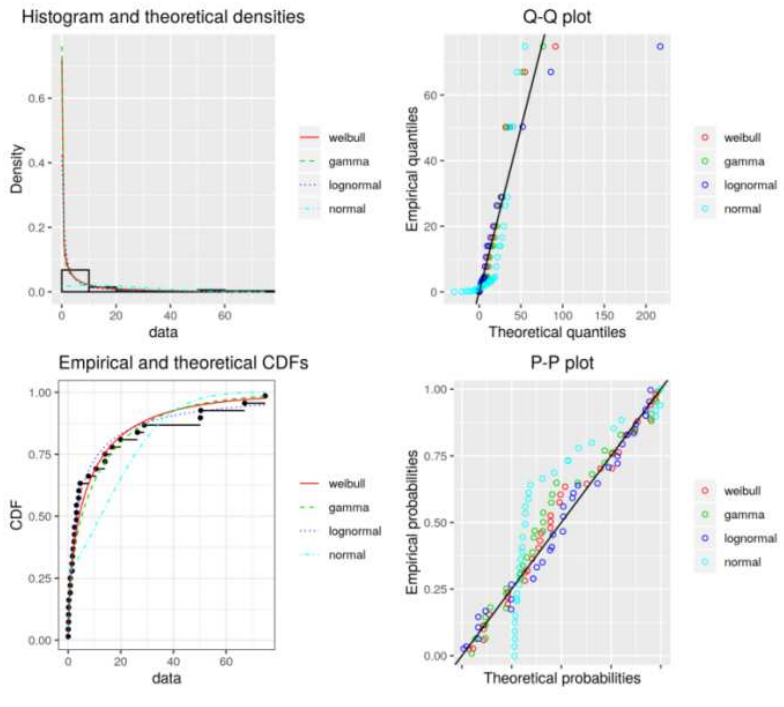


Figure 9: Data and fitting distribution for AFs in feed

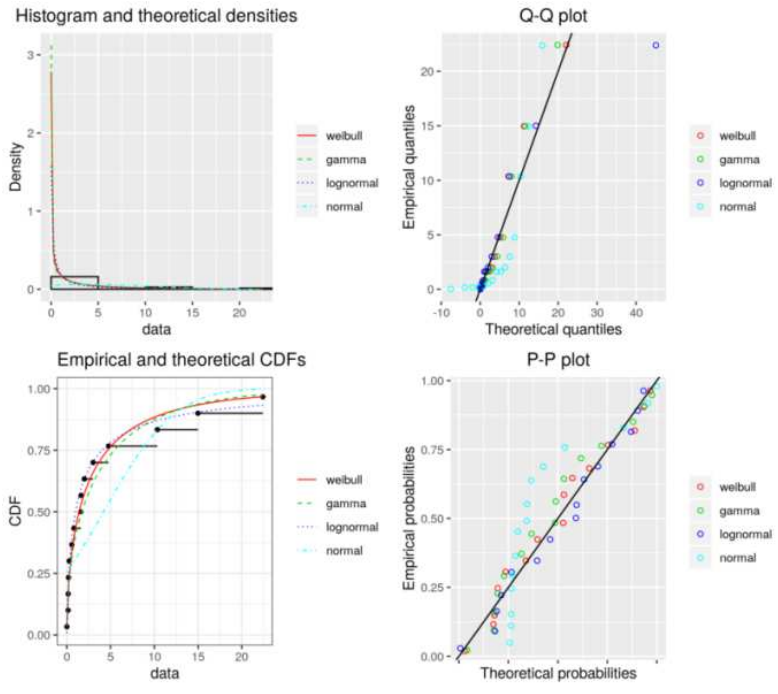


Figure 10: Data and fitting distribution for AFs in food

2.1.3.2. EFSA data

The distribution of occurrence data per mycotoxin in feed and food has been compared to the data distribution of the corresponding theoretical model, and illustrated in the figures below. Data and fitting distributions for other mycotoxins are shown in Appendix B.

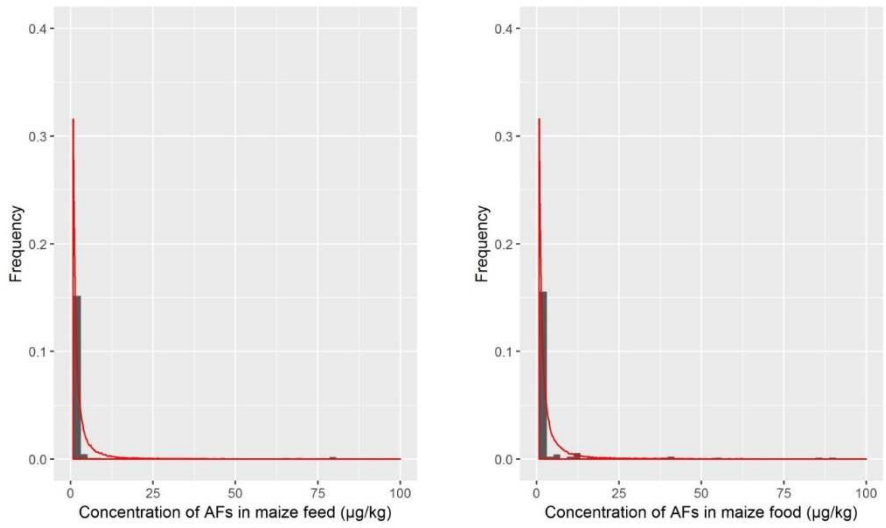


Figure 11 (a,b) : Data distribution of total aflatoxins in maize feed and food from EFSA database compared to the fitting distribution (red line).

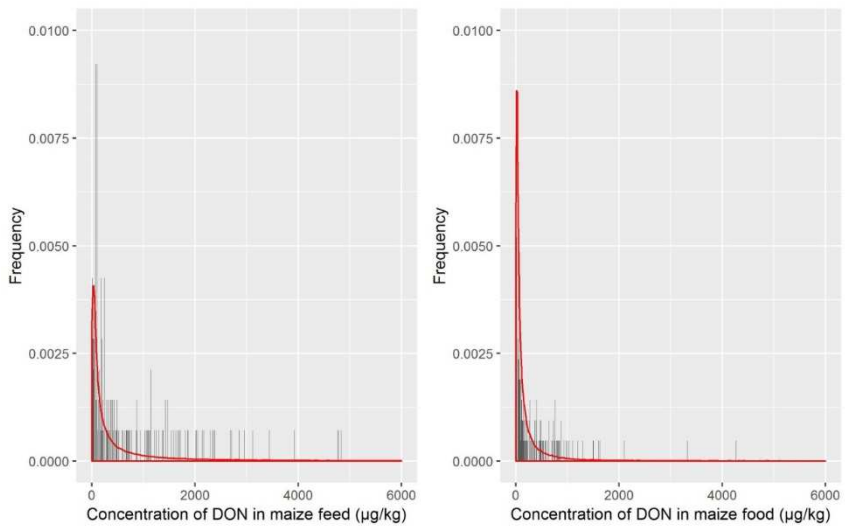


Figure 12 (a,b): Data distribution of DON in maize feed and food from EFSA database compared to the fitting distribution (red line).

2.1.4. *Co-occurrence estimate: probabilities of co-occurrence by multinomial* distribution analysis

2.1.4.1. Literature data

In the context of co-occurrence of mycotoxin native forms, the frequency in which a mycotoxin was reported alone (i.e. AFs, FBs, etc.) or in combination with others was recorded, allowing the identification of patterns of co-occurrence and their frequency for each dataset. The former was used to fit a multinomial model to estimate the probability of each mycotoxin being present in a food or feed sample. Estimation of such probability was performed using a multinomial model using frequencies of each combination of mycotoxin which was then simulated to estimate potential co-occurrence based on the observed patterns reported. In Literature data, four different patterns have been observed in maize out of eight possible combinations. A total number of 106 observations were obtained for the four patters. Number of observations counted for each pattern is reported in Table 7.

Table 7: Total number of observations with specific pattern of co-occurrence

Patterns	AFs	DON	FBs	N
1	1	1	1	1
2	0	1	1	81
3	0	0	1	9
4	0	1	0	15

N: Number of total observations for each pattern

The multinomial model estimated the probabilities of the mycotoxins to co-occur. As shown in Table 8, DON and FBs have the highest simulated probability to co-occur (74,4 %), whereas the probability of AFs in co-occurrence either with FB and DON is quite low, ranging between 0,5 % and 0,3 %, respectively. The probability of finding all three mycotoxins together is also low (1,0 %).

Table 8: Probability simulated by the multinomial model of having co-occurring mycotoxins in maize using Literature occurrence dataset

Patterns	AFs	DON	FBs	Simulated probability
1			1	10,7 %
2	1		1	0,5 %
3		1		13,1 %
4	1	1		0,3 %

5		1	1	74,4 %
6	1	1	1	1,0 %

2.1.4.2. EFSA data

Forty-seven patterns of co-occurring mycotoxins have emerged from EFSA data for a total of 3143 observations (Table 9).

Table 9: Total number of observations with specific pattern of co-occurrence

Pattern	AFs	DON	FBs	OTA	T2+HT2	ZEN	N
1	0	0	0	0	0	1	391
2	0	0	1	0	0	1	103
3	0	0	1	0	0	0	534
4	0	0	0	1	0	0	73
5	1	0	1	0	0	1	75
6	0	0	1	1	0	0	10
7	1	1	1	1	1	1	2
8	1	1	1	0	0	1	27
9	0	1	1	1	0	1	46
10	1	0	1	0	0	0	104
11	0	1	1	0	0	1	28
12	1	0	0	0	0	0	739
13	0	1	0	0	0	1	49
14	1	1	1	0	0	0	9
15	1	0	0	0	0	1	36
16	0	1	0	0	0	0	234
17	1	0	1	1	0	0	16
18	1	0	0	1	0	0	18
19	0	0	1	1	0	1	1
20	0	0	1	0	1	0	56
21	1	1	1	1	0	1	36
22	0	1	0	1	0	1	10
23	0	0	0	1	0	1	7
24	1	1	1	0	1	1	30
25	0	1	1	1	1	1	32
26	0	1	1	0	0	0	28
27	0	0	0	0	1	0	158
28	0	1	1	0	1	1	71
29	1	0	1	0	1	1	22
30	0	0	1	0	1	1	10
31	0	1	0	0	1	0	3

32	0	1	1	0	1	0	3
33	1	0	0	0	1	1	15
34	0	1	0	1	0	0	3
35	1	1	0	0	0	0	16
36	0	1	1	1	0	0	3
37	0	0	0	1	1	0	1
38	1	1	0	1	1	1	8
39	1	1	0	1	0	1	6
40	1	0	0	1	0	1	9
41	0	0	0	0	1	1	6
42	1	0	0	0	1	0	4
43	1	0	0	1	1	0	1
44	1	1	0	0	0	1	8
45	1	0	1	1	0	1	87
46	1	1	0	0	1	1	14
47	0	1	0	0	1	1	1

N: Number of total observations for each pattern

The probabilities for AF, DON, FB, OTA, T2/HT2 and ZEN to co-occur in maize are reported in Table 10. It was decided to include only six mycotoxins in the multinomial analysis because for the remaining mycotoxins information was very limited, therefore modelling was not simple.

Table 10: Probability simulated by the multinomial model of having co-occurring mycotoxins in maize using EFSA occurrence dataset

Pattern	AFs	DON	FBs	OTA	T2+HT2	ZEN	%
1				1	1	1	0,30
2			1			1	1,20
3			1		1		0,10
4			1		1	1	2,20
5			1	1			0,10
6			1	1		1	1,40
7			1	1	1		0,40
8			1	1	1	1	1,90
9		1				1	0,10
10		1			1	1	0,60
11		1		1		1	0,50
12		1		1	1	1	0,20
13		1	1				0,40
14		1	1			1	3,10
15		1	1		1		0,40

16		1	1		1	1	2,20
17		1	1	1			0,50
18		1	1	1		1	2,30
19		1	1	1	1		1,00
20		1	1	1	1	1	1,40
21	1					1	0,90
22	1				1	1	1,00
23	1			1		1	1,30
24	1			1	1	1	1,20
25	1		1				1,60
26	1		1			1	9,60
27	1		1		1		2,10
28	1		1		1	1	6,60
29	1		1	1			2,30
30	1		1	1		1	4,90
31	1		1	1	1		1,20
32	1		1	1	1	1	3,00
33	1	1					0,10
34	1	1				1	3,00
35	1	1			1		0,40
36	1	1			1	1	2,30
37	1	1		1			0,20
38	1	1		1		1	2,00
39	1	1		1	1		0,80
40	1	1		1	1	1	1,00
41	1	1	1				4,00
42	1	1	1			1	13,30
43	1	1	1		1		3,30
44	1	1	1		1	1	4,00
45	1	1	1	1			2,40
46	1	1	1	1		1	4,40
47	1	1	1	1	1		1,90
48	1	1	1	1	1	1	0,90

2.1.5. Occurrence data considered for dietary exposure assessment

Criteria to include mycotoxins in dietary exposure assessment:

- Highly reported mycotoxins in maize: availability of sufficient occurrence data;

- Characterization of the mixture: patterns of co-occurrence observed by co-occurrence analysis.

Considering the results obtained from the qualitative and quantitative analysis of occurrence data and the observed pattern of co-occurring mycotoxins, it was considered adequate for dietary exposure assessment to include AFs, FBs and DON from Literature data and AFs, FBs, DON, OTA, T2/HT2 and ZEN for EFSA data. The text below describes in more details the occurrence data for these mycotoxins.

2.1.5.1. Feed occurrence data considered for dietary exposure

Table 11 provides a summary of occurrence data in feed as reported in Literature and EFSA databases including statistical descriptors of the results (mean and P95) using max LOD/LOQ and mean LOD/LOQ approach.

Table 11: Statistical description of the occurrence of mycotoxins in maize based **feed** across Literature and EFSA data using max and mean LOD/LOQ (µg/kg)

	Max				**Mean**			
	LB		**UB**		**LB**		**UB**	
	Mean	**P95**	**Mean**	**P95**	**Mean**	**P95**	**Mean**	**P95**
Literature								
AF	0.188	0	10.2	10	0.225	0	1,5	1.3
FB	1699.7	7955.8	1961.3	7955.8	1733.2	7955.8	1854	7955.8
DON	735.9	2563.1	835.4	2563.1	773.2	2563.1	790.9	2563.1
EFSA								
AF	0.14	0	10.5	10	0.766	1.3	2.3	6.1
FB	185.9	1242.5	529.8	1242.5	206.4	1242.5	448.5	1242.5
DON	441.3	2098.7	548.7	2098.7	460.1	2098.7	495.9	2098.7
OTA	0.104	0.6	0.821	2	0.187	1	0.549	1
T2+HT2	1.8	5	26.4	100	5.8	35.9	8.7	35.9
ZEN	37.7	256.9	111.1	256.9	49.2	256.9	71.7	256.9

In **Literature**, the highest mean concentrations were observed in FBs (mean LB/UB ranged from 1,699 to 1,961µg/kg). These results are in agreement with results already described, being maize one of the most contaminated cereal by FBs (EFSA,

2018c). DON and AFs mean LB/UB concentration ranged from 735 to 835 μg/kg and 0.188 to10.2 μg/kg, respectively. In **EFSA data**, the lowest concentrations of FBs were observed compared to Literature (mean LB/UB ranged from 185.9 to 529.8 μg/kg). Whereas, the highest mean concentrations were observed in DON (mean LB/UB ranged from 441.3 to 548.7 μg/kg). Mean LB/UB of AFs ranged from 0.14 to 10.5 μg/kg.

Overall, the highest LB mean concentrations were observed when using mean values of LOD/LOQ compared to max values. The reason for this is the increase of analytical results below the LOD and thereby substituted by 0 when considering higher values of LOD/LOQ, which lower the total mean concentration. The number of analytical results below (True) and above (False) LOD and LOQ in the two different approaches is provided in Table 12 and Table 13.

Table 12: Number of analytical results above and below LOD and LOQ in feed – Literature data

	AFs		**FBs**		**DON**	
	False	**True**	**False**	**True**	**False**	**True**
Max LOD	35	965	597	403	842	158
Mean LOD	63	937	634	366	845	155
Max LOQ	967	33	799	201	730	270
Mean LOQ	982	18	827	173	888	112

False: above LOD/LOQ; True: below LOD/LOQ

Table 13: Number of analytical results above and below LOD and LOQ in feed – EFSA data

	AFs		**FBs**		**DON**		**OTA**		**T2/HT2**		**ZEN**	
	F	**T**	**F**	**T**	**F**	**T**	**F**	**T**	**F**	**T**	**F**	**T**
Max LOD	21	979	251	749	677	323	157	843	213	787	74	926
Mean LOD	152	848	291	709	690	310	284	716	247	753	74	926
Max LOQ	980	20	827	173	601	399	850	150	791	209	936	64
Mean LOQ	887	113	828	172	728	272	814	186	914	86	947	53

False (F): above LOD/LOQ; True (T): below LOD/LOQ

2.1.5.2. Food occurrence data considered for dietary exposure

Table 14 provides a summary of mycotoxin occurrence in food including statistical descriptors of the results (mean and P95) using max and mean LOD/LOQ approaches. In **Literature**, the mean concentrations of AFs, FBs and DON ranged from 0.134 to 10.2 μg/kg (LB/UB), 128.9 to 475.8 μg/kg (LB/UB) and 280.3 to 479.3 μg/kg, respectively. In **EFSA data**, the mean concentration of AFs, FBs and DON ranged from 0.270 to 10.8 μg/kg (LB/UB), 155.2/498.2 μg/kg (LB/UB) and 222.1/370 μg/kg (LB/UB), respectively. With regards to OTA, T2+HT2 and ZEN, concentrations could be obtained only from EFSA datasets; mean concentrations ranged from 0.132/0.842, 0.732/12.8 and 9/27.7 μg/kg (LB/UB), respectively.

Table 14: Statistical description of the concentrations of mycotoxins in maize based **food** across Literature and EFSA using the max and mean LOD/LOQ values (μg/kg)

	Max				**Mean**			
	LB		**UB**		**LB**		**UB**	
	Mean	**P95**	**Mean**	**P95**	**Mean**	**P95**	**Mean**	**P95**
Literature								
AF	0.134	0	10.2	10.0	0.210	0	1.6	1.3
FB	128.9	300.0	475.8	1000.0	146.7	962.7	397.8	962.7
DON	280.3	1389.1	479.3	1389.1	333.1	1389.1	376.3	1389.1
EFSA								
AF	0.270	0	10.8	10.0	1.1	1.3	2.8	6.1
FB	116.2	300.0	451.3	1000.0	130.5	901.8	380.1	901.8
DON	80.9	532.1	200.6	532.1	98.5	532.1	141.5	532.1
OTA	0.132	0.6	0.842	2.0	0.213	1.3	0.565	1.3
T2+HT2	0.732	5.0	12.8	100	1.4	3.0	4.6	10.0
ZEN	18.3	10.0	87.6	200.0	25.2	137.9	49.5	137.9

Overall, slightly higher concentrations were observed in EFSA datasets for AFs compared to Literature; whereas higher concentrations of FBs and DON were reported in Literature. Furthermore, greater mean concentrations were observed when using mean values of LOD/LOQ compared to max values. And the reason for this is that, when using greater values of LOD/LOQ, the number of analytical results below the LOD and, therefore, replaced by 0, increases lowering the mean concentration. The number of analytical results above (False) and below (True) LOD and LOQ in the two different approaches is provided in Table 15 and Table 16.

Table 15: Number of analytical results above and below LOD and LOQ in food - Literature data

	AFs		FBs		DON	
	F	T	F	T	F	T
Max LOD	6	994	207	793	828	172
Mean LOD	45	955	259	741	836	164
Max LOQ	994	6	847	153	421	579
Mean LOQ	969	31	829	171	686	314

False (F): above LOD/LOQ; True (T): below LOD/LOQ

Table 16: Number of analytical results above and below LOD and LOQ in food - EFSA data

	AFs		FBs		DON		OTA		T2/HT2		ZEN	
	F	T	F	T	F	T	F	T	F	T	F	T
Max LOD	31	969	178	822	585	415	159	841	80	920	62	938
Mean LOD	192	808	220	780	618	382	226	774	114	886	62	938
Max LOQ	971	29	870	130	543	457	858	142	924	76	946	54
Mean LOQ	856	144	853	147	645	355	884	116	932	68	959	41

False (F): above LOD/LOQ; True (T): below LOD/LOQ

2.2. Summary of exposure estimates

2.2.1. Animals

For all the animal categories, P95 and mean exposures have been estimated based on the 95th percentile and the LB/UB mean concentrations, respectively. The following paragraphs provide a description of exposure estimates per each class of mycotoxin in animals.

AFs: Estimates of mean and P95 exposures of chronic exposure to the sum of AFs in pigs and poultry are given in Table 17. The highest estimated exposure was observed in fattening chickens in both EFSA and Literature datasets, with mean dietary exposure ranging between 0.016/0.814 and 0.015/0.806 μg/kg bw per day (LB/UB), respectively. Mean dietary exposure for piglets and fattening pigs were higher in EFSA dataset being 0.013/0.474 μg/kg bw per day (LB/UB) and

0.007/0.378 μg/kg bw per day (LB/UB), respectively. Overall, exposure metrics are similar in the two datasets.

Table 17: Estimates of P95 and mean exposure to AFs for pigs and poultry derived from LB and UB concentrations from Literature and EFSA data

Animal species	LB/UB	Exposure μg/kg bw per day							
		Literature				EFSA			
		Max LOD/LOQ		Mean LOD/LOQ		Max LOD/LOQ		Mean LOD/LOQ	
		Mean	P95	Mean	P95	Mean	P95	Mean	P95
Piglets	LB	0.008	0	0.010	0	0.013	0	0.032	0.057
	UB	0.449	0.440	0.068	0.057	0.474	0.440	0.104	0.268
Fattening pigs	LB	0.007	0	0.008	0	0.007	0	0.028	0.048
	UB	0.374	0.367	0.057	0.048	0.378	0.367	0.084	0.224
Fattening chickens	LB	0.015	0	0.018	0	0.016	0	0.061	0.103
	UB	0.806	0.790	0.122	0.103	0.814	0.790	0.182	0.482
Laying hens	LB	0.010	0	0.012	0	0.010	0	0.041	0.069
	UB	0.541	0.530	0.082	0.069	0.546	0.530	0.122	0.323

bw: body weight; LB: lower bound; UB: upper bound

DON: Concentrations of the sum of DON, 3-Ac-DON, 15-Ac-DON and DON-3-glucoside in maize-based feed materials were used to estimate the mean and 95th percentile exposures by pigs and poultry (Table 18). The highest estimated exposure was for fattening chickens in both EFSA and Literature datasets, with LB and UB estimates of 34.8/43.3 and 58.1/66.0 μg/kg bw per day, respectively, at the mean level. Exposures estimates for piglets and fattening pigs ranged from 19.4/36.8 and 16,2/30.6 μg/kg bw per day, respectively, at the mean level. Similar results were presented by EFSA assessment in 2017, where the highest level of exposure to DON and its metabolites were also observed in fattening chickens and in laying hens (61.0/62.0 and 47.6/48.8 μg/kg bw per day for LB and UB, respectively, at the mean level) (EFSA, 2017c). Whereas, exposures for pigs were lower for piglets and fattening pigs in last EFSA assessment, i.e. 12.5/20.5 and 13.1/15.1 μg/kg bw per day (LB/UB), respectively, at the mean level.

Table 18: Estimates of P95 and mean exposure to DON and its metabolites for pigs and poultry derived from LB and UB concentrations from Literature and EFSA data

Animal species	LB/UB	Exposure μg/kg bw per day	
		Literature	EFSA

		Max LOD/LOQ		Mean LOD/LOQ		Max LOD/LOQ		Mean LOD/LOQ	
		Mean	**P95**	**Mean**	**P95**	**Mean**	**P95**	**Mean**	**P95**
Piglets	LB	32.4	112.8	34.0	112.8	19.4	92.3	20.2	92.3
	UB	36.8	112.8	34.8	112.8	24.1	92.3	21.8	92.3
Fattening pigs	LB	27.0	94.0	28.4	94.0	16,2	77.0	16.9	76.9
	UB	30.6	94.0	29.0	94.0	20,1	77.0	18.2	76.9
Fattening chickens	LB	58.1	202.5	61.1	202.5	34.8	165.8	36.3	165.8
	UB	66.0	202.5	62.5	202.5	43.3	165.8	39.2	165.8
Laying hens	LB	39.0	135.8	41.0	135.8	23.4	111.2	24.3	111.2
	UB	44.3	135.8	41.9	135.8	29.1	111.2	26.3	111.2

bw: body weight; LB: lower bound; UB: upper bound

FBs: Estimates of mean and P95 exposures of chronic exposure to the sum of FBs for pigs and poultry, are given in Table 19. The highest mean dietary exposure to FBs was observed in fattening chickens in both EFSA and Literature datasets, and ranged from 14.0/41.9 to 134.3/155.0 (LB/UB) μg/kg bw per day, respectively. Mean exposures for laying hens were only marginally lower (9.9/28.1 and 90.1/104.0 (LB/UB) μg/kg bw per day); for piglets and fattening pigs mean exposures ranged from 8.2/23.3 to 6.8/19.4 (LB/UB) μg/kg bw per day, respectively. It should be noted that there is consistent difference between exposures from the two datasets, being the higher FB occurrence reported in Literature. In EFSA opinion 2014, the highest level of exposure to FBs were also observed in fattening chickens and in laying hens (22.1/34.5 and 19.9/33.4 μg/kg bw per day for LB and UB, respectively, at the mean level). It should also be noted that at the 95th percentile exposure no change was observed in the LB/UB scenario. This is because, as described in section 4.1.5, the number of LCD was limited for this mycotoxin and the curve of the estimated distribution was very skewed; thus the amount of quantified data was above the 5% and the p95 was in that 5% quantified data, and therefore it didn't change in the LB and UB scenario.

Table 19: Estimates of P95 and mean exposure to FBs for pigs and poultry derived from LB and UB concentrations from Literature and EFSA data

Animal species	**LB/UB**	**Exposure μg/kg bw per day**							
		Literature				**EFSA**			
		Max LOD/LOQ		**Mean LOD/LOQ**		**Max LOD/LOQ**		**Mean LOD/LOQ**	
		Mean	**P95**	**Mean**	**P95**	**Mean**	**P95**	**Mean**	**P95**

Piglets	LB	74.8	350.1	77.8	350.1	8.2	54.7	9.1	54.7
	UB	86.3	350.1	81.6	350.1	23.3	54.7	19.7	54.7
Fattening	LB	62.3	291.7	64.8	291.7	6.8	45.6	7.5	45.5
pigs	UB	71.9	291.7	68.0	291.7	19.4	45.6	16.5	45.5
Fattening	LB	134.3	628.5	139.7	628.5	14.0	98.2	16.3	98.2
chickens	UB	155.0	628.5	146.5	628.5	41.9	98.2	35.4	98.2
Laying	LB	90.1	421.7	93.7	421.7	9.9	65.9	10.9	65.8
hens	UB	104.0	421.7	98.3	421.7	28.1	65.9	23.7	65.8

bw: body weight; LB: lower bound; UB: upper bound

OTA: Estimates of mean and P95 exposures of chronic exposure by pigs and poultry to the OTA were calculated using only EFSA occurrence data because no sufficient occurrence data was retrieved from Literature to allow risk assessment (Table 20). The highest estimated exposure was for fattening chickens, with LB and UB estimates of 0.008/0.065 μg/kg bw per day, at the mean level. The lowest exposures were observed in fattening pigs (0.004/0.030 μg/kg bw per day, at the mean level (LB/UB)).

Table 20: Estimates of P95 and mean exposure to OTA for pigs and poultry derived from LB and UB concentrations from EFSA data

Animal species	LB/UB	Exposure μg/kg bw per day			
		EFSA			
		Max LOD/LOQ		Mean LOD/LOQ	
		Mean	P95	Mean	P95
Piglets	LB	0.005	0.026	0.008	0.044
	UB	0.036	0.088	0.024	0.044
Fattening pigs	LB	0.004	0.022	0.007	0.037
	UB	0.030	0.073	0.020	0.037
Fattening chickens	LB	0.008	0.047	0.015	0.079
	UB	0.065	0.158	0.043	0.079
Laying hens	LB	0.006	0.032	0.010	0.053
	UB	0.044	0.106	0.029	0.053

bw: body weight; LB: lower bound; UB: upper bound

T2/HT2: In 2011, EFSA derived a LOAEL for the sum of T2 and HT2 of 29 and 40 μg/kg bw per day for pigs and poultry, respectively. In **pigs**, the mean dietary exposure to T2 and HT2 ranged from 0.066 (lowest LB) to 0.986 (highest UB) μg/kg bw per day. At the 95th percentile the exposure was estimated to be 4.4 and 3.7 (highest UB) μg/kg bw per day for piglets and fattening pigs, respectively. This is

up to 15 % of the LOAEL and not of concern. In **poultry**, the highest estimated exposure to T2 and HT2 toxin was in chicken for fattening with 0.687 (mean) and 7.9 (95th percentile) μg/kg bw per day. This is up to 20 % of the LOAEL and not of concern.

Table 21: Estimates of P95 and mean exposure to T2/HT2 for pigs and poultry derived from LB and UB concentrations from EFSA data

Animal species	**LB/UB**	**Exposure μg/kg bw per day**			
		EFSA			
		Max LOD/LOQ		**Mean LOD/LOQ**	
		Mean	**P95**	**Mean**	**P95**
Piglets	LB	0.079	0.220	0.255	1.6
	UB	1.2	4.4	0.383	1.6
Fattening pigs	LB	0.066	0.183	0.213	1.3
	UB	0.968	3.7	0.319	1.3
Fattening chickens	LB	0.142	0.395	0.458	2.8
	UB	2.1	7.9	0.687	2.8
Laying hens	LB	0.095	0.265	0.307	1.9
	UB	1.4	5.3	0.461	1.9

bw: body weight; LB: lower bound; UB: upper bound

ZEN: In **pig**, the mean dietary exposure to ZEN ranged from 1.4 (lowest LB) to 4.9 (highest UB) μg/kg bw per day. This is up to 47 % of the NOEL of 10.4 μg/kg bw per day and not of concern. However, at the 95th percentile the exposure was estimated to be 11.3 (highest UB) μg/kg bw per day for piglets which is above the NOEL. In **poultry**, the highest estimated exposure to ZEN was in chicken for fattening with a maximum of 20.3 (95th percentile) μg/kg bw per day. This is up to 0,2 % of the NOAEL of 7,500 μg/kg bw per day and not of concern.

Table 22: Estimates of P95 and mean exposure to ZEN and its metabolites for pigs and poultry derived from LB and UB concentrations from EFSA data

Animal species	**LB/UB**	**Exposure μg/kg bw per day**			
		EFSA			
		Max LOD/LOQ		**Mean LOD/LOQ**	
		Mean	**P95**	**Mean**	**P95**
Piglets	LB	1.7	11.3	2.2	11.3
	UB	4.9	11.3	3.2	11.3

Fattening pigs	LB	1.4	9.4	1.8	9.4
	UB	4.1	9.4	2.6	9.4
Fattening chickens	LB	2.9	20.3	3.9	20.3
	UB	8.8	20.3	5.6	20.3
Laying hens	LB	1.9	13.6	2.6	13.6
	UB	5.9	13.6	3.8	13.6

bw: body weight; LB: lower bound; UB: upper bound

2.2.1.1. Conclusion

Overall, the highest exposures for multiple mycotoxins were observed in chicken. No significant differences were observed amongst the two datasets (i.e. EFSA and Literature) with the exception of FBs. All LB/UB mean and P95 were below the TDI identified from EFSA opinions and the OpenFoodTox database., with the only exception of ZEN in piglets.

2.2.2. *Humans*

P95 and mean chronic dietary exposures have been estimated based on 95th percentiles and the mean LB and UB concentrations across age classes (Table 23, Table 24, Table 25, Table 26 and Table 27). Therefore, reported exposure metrics at P95 do not refer to high consumers but rather to highly contaminated food.

AFs: For mean dietary exposure, the highest estimated chronic dietary exposures to AFs were observed in adults (0.102/0.999 ng/kg bw per day (LB/UB)). The P95 dietary exposure, ranged from 0 (lowest LB) to 0.925 (highest UB) μg/kg bw per day across age groups and datasets. Overall, exposure metrics are very similar across the literature and EFSA datasets but are slightly higher in the EFSA datasets for which higher AF concentrations were observed.

Table 23: Summary of exposure estimates to **AFs (ng/kg bw/day)** across EFSA and Literature datasets

		max LOD/LOQ				**mean LOD/LOQ**			
		LB		**UB**		**LB**		**UB**	
		Mean	**P95**	**Mean**	**P95**	**Mean**	**P95**	**Mean**	**P95**
AF	**Adolescent**								
	Literature	0.008	0	0.613	0.601	0.013	0	0.096	0.078
	EFSA	0.016	0	0.649	0.601	0.066	0.078	0.168	0.367
	Adults								
	Literature	0.012	0	0.944	0.925	0.019	0	0.148	0.120
	EFSA	0.025	0	0.999	0.925	0.102	0.120	0.259	0.564
	Elderly								
	Literature	0.006	0	0.464	0.455	0.010	0	0.073	0.059
	EFSA	0.012	0	0.491	0.455	0.050	0.059	0.127	0.277

FBs: The highest estimated chronic dietary exposure to FBs was observed in adults. For mean dietary exposure, the highest estimated LB exposure levels were in order adults, adolescents and elderly with a maximum of 0.012 μg/kg bw per day. The highest UB exposure was also observed in adults 0.042 μg/kg bw per day. The P95 dietary exposure, ranged from 0.014 (lowest LB) to 0.093 (highest UB) μg/kg bw per day across age groups and datasets. Overall, exposure metrics are very similar across EFSA and Literature datasets but slightly higher in the former where higher FB concentrations were observed. All LB/UB mean were below the provisional maximum TDI (PMTDI) for FBs of 2 μg/kg bw per day (EFSA, 2014a).

Table 24: Summary of exposure estimates to **FBs (μg/kg bw/day)** across EFSA and Literature datasets

		max LOD/LOQ				mean LOD/LOQ			
		LB		UB		LB		UB	
		Mean	P95	Mean	P95	Mean	P95	Mean	P95
FBs	**Adolescent**								
	Literature	0.008	0.018	0.029	0.060	0.009	0.058	0.024	0.058
	EFSA	0.007	0.018	0.027	0.060	0.008	0.054	0.023	0.054
	Adults								
	Literature	0.012	0.028	0.044	0.093	0.014	0.089	0.037	0.089
	EFSA	0.011	0.028	0.042	0.093	0.012	0.083	0.035	0.083
	Elderly								
	Literature	0.006	0.014	0.022	0.045	0.007	0.044	0.018	0.044
	EFSA	0.005	0.014	0.021	0.045	0.006	0.041	0.017	0.041

DON: The mean dietary exposure to DON ranged from 0.004 (lowest LB) to 0.044 (highest UB) μg/kg bw per day across age groups and datasets. The P95 dietary exposure, ranged from 0.024 (lowest LB) to 0.128 (highest UB) μg/kg bw per day across age groups and datasets. The highest estimated chronic dietary exposures to DON were observed in adults. Overall, exposure metrics are very similar across EFSA and Literature datasets but slightly higher in the latter where higher DON concentrations were observed. All LB/UB mean were below the TDI for DON of 1 μg/kg bw per day (EFSA, 2017c).

Table 25: Summary of exposure estimates to **DON (μg/kg bw/day)** across EFSA and Literature datasets

		max LOD/LOQ				mean LOD/LOQ			
		LB		UB		LB		UB	
		Mean	P95	Mean	P95	Mean	P95	Mean	P95
DON	**Adolescent**								
	Literature	0.017	0.083	0.029	0.083	0.020	0.083	0.023	0.083
	EFSA	0.005	0.032	0.012	0.032	0.006	0.032	0.009	0.032
	Adults								
	Literature	0.026	0.128	0.044	0.128	0.031	0.128	0.035	0.128
	EFSA	0.007	0.049	0.019	0.049	0.009	0.049	0.013	0.049
	Elderly								
	Literature	0.013	0.063	0.022	0.063	0.015	0.063	0.017	0.063
	EFSA	0.004	0.024	0.009	0.024	0.004	0.024	0.006	0.024

OTA: For mean dietary exposure, the highest estimated LB exposure levels were in order adults, adolescents and elderly with a maximum of 0.02 ng/kg bw per day. The highest UB exposure was also observed in adults 0.078 μg/kg bw per day. The P95 dietary exposure, ranged from 0.027 (lowest LB) to 0.185 (highest UB) μg/kg bw per day across age groups. In the upcoming EFSA opinion on OTA, the mean LB/UB exposures range between 1.30/4.53 (mean) and 3.16/8.07 (P95) ng/kg bw per day across the three age groups and considering a full diet. Thus, comparing the two results we can observe that the exposure to OTA in human from a maize-based diet is about 50 times below the exposures caused by a full-diet.

Table 26: Summary of exposure estimates to **OTA (ng/kg bw/day)** in EFSA dataset

	OTA							
	max LOD/LOQ				**mean LOD/LOQ**			
	LB		**UB**		**LB**		**UB**	
	Mean	**P95**	**Mean**	**P95**	**Mean**	**P95**	**Mean**	**P95**
Adolescent	0.008	0.036	0.051	0.120	0.013	0.078	0.034	0.078
Adults	0.012	0.056	0.078	0.185	0.020	0.120	0.052	0.120
Elderly	0.006	0.027	0.038	0.091	0.010	0.059	0.026	0.059

ZEN: The mean dietary exposure to ZEN ranged from 0.00083 (lowest LB) to 0.00810 (highest UB) μg/kg bw per day across age groups. The P95 dietary exposure, ranged from 0.00045 (lowest LB) to 0.01850 (highest UB) μg/kg bw per day across age groups and datasets. The highest estimated chronic dietary exposures to ZEN were observed in adults. All LB/UB mean were below the TDI for ZEN of 0.25 μg/kg bw per day (EFSA, 2016b).

T2/HT2: The mean dietary exposure to T2 and HT2 ranged from 0.00003 (lowest LB) to 0.00118 (highest UB) μg/kg bw per day across age groups. The TDI for the sum of T2 and HT2 is 0.2 μg/kg bw per day (200 ng/kg bw per day) (EFSA, 2016a). Our exposures are far below the TDI, thus the exposure to the sum of T2 and HT2 through maize based food is not considered to be of concern.

Table 27: Summary of exposure estimates to **T2/HT2** and **ZEN (μg/kg bw/day)** in EFSA dataset

	max LOD/LOQ				**mean LOD/LOQ**			
	LB		**UB**		**LB**		**UB**	
	Mean	**P95**	**Mean**	**P95**	**Mean**	**P95**	**Mean**	**P95**
	Adolescent							
T2+HT2	0.00004	0.00030	0.00077	0.00601	0.00008	0.00018	0.00028	0.00060
ZEN	0.00110	0.00060	0.00526	0.01202	0.00151	0.00829	0.00297	0.00829
b)	**Adults**							
T2+HT2	0.00007	0.00046	0.00118	0.00925	0.00013	0.00028	0.00043	0.00093
ZEN	0.00169	0.00093	0.00810	0.01850	0.00233	0.01276	0.00458	0.01276
c)	**Elderly**							
T2+HT2	0.00003	0.00023	0.00058	0.00455	0.00006	0.00014	0.00021	0.00045
ZEN	0.00083	0.00045	0.00398	0.00909	0.00115	0.00627	0.00225	0.00627

2.2.2.1. Conclusion

Overall, the highest exposure metrics were observed for adults > adolescents > elderly across the individual mycotoxins accordingly to mean consumption rates. No significant differences were observed for AFs, FBs and DON among the two datasets (i.e. EFSA and Literature). All LB/UB mean and P95 were below the TDI identified in EFSA opinion.

2.3. *Hazard identification and characterisation*

Toxicological end points were identified by EFSA CONTAM Panel and were used to characterise human health and animal health risk associated with chronic exposure to multiple mycotoxins. Based on the data collected, assessment groups were set. Further details are provided in the following paragraphs for each mycotoxin in the assessment groups and summarised in Table 28.

2.3.1. *Aflatoxins*

The EFSA CONTAM Panel evaluated the mycotoxin aflatoxin B1 and derived a **$BMDL_{10}$ 0.4 of µg/kg bw/day** based on benchmark dose (BMD) analysis using model averaging of the incidence of hepatocellular carcinomas (HCC) in male Fisher rats following AFB1 exposure (Wogan et al., 1974; EFSA et al., 2017). This $BMDL_{10}$ was used as a reference point for the risk characterisation of AF in the last EFSA opinion available online for public consultation[2]. The CONTAM Panel also stated that calculation of a BMDL from the human data was not appropriate; instead, the cancer potencies estimated by JECFA in 2016 were used. Following respective EFSA guidance on substances which are both genotoxic and carcinogenic a **MOE of ≥ 10,000** would be considered of low concern for neoplastic risks in humans (EFSA, 2005a).

2.3.2. *Fumonisins*

The toxicity of FBs has been largely demonstrated in several animal species, and the International Agency for Research on Cancer (IARC) have classified them in group 2B carcinogens. FBs are poorly absorbed from the gastrointestinal tract and their absorbed fractions are rapidly excreted, mainly in the bile of experimental animals (approximately 1.4% of the dose), resulting in low tissue concentrations (Dantzer et al., 1999; Martinez-Larranaga et al., 1999). FB1 is considered not to be acutely toxic, but in repeated dose studies with rodents, it causes liver and kidney toxicity (Marin et al., 2013).

Since 2005, EFSA has assessed risk of dietary exposure to FBs and has identified reference points for both human and animal risk assessment (EFSA, 2005b, 2014a, 2018a, c). Regarding **pigs,** a LOAEL of 200 µg/kg bw per day of FBs (based on FB1) was derived by EFSA in 2005 (EFSA, 2005b). This was based on the accumulation in sphingoid bases in serum and tissue organs in pigs given FB-contaminated feeds (Riley et al., 1993). Subsequently, EFSA reviewed several *in vivo* pig experiments

[2] http://www.efsa.europa.eu/it/consultations/call/public-consultation-draft-scientific-opinion-risks

and confirmed that exposure to FBs disturb the sphinganine/ sphingosine (Sa/So) ratio in blood and tissues, and induces specific syndromes for FB1–3 toxicity such as pulmonary oedema, lung and hepatic lesions (Zomborszky-Kovacs et al., 2002a; Zomborszky-Kovacs et al., 2002b). From this evidence, EFSA identified a **NOAEL of 1 mg FB1/kg feed** (corresponding to 40 μg/kg bw per day) (EFSA, 2018c). Regarding **poultry,** EFSA derived a **NOAEL of 20 mg/kg feed (corresponding to 2 mg/kg bw per day)**. This was based on the decrease of total liver lipids in chickens given 40 mg FB/kg feed (Henry et al., 2000; EFSA, 2018c). Regarding **human**, in 2018, EFSA evaluated FB1 and derived a **$BMDL_{10}$ of 0.1 mg/kg bw per day** based on non-neoplastic hepatotoxicity in mice with a critical effect associated with an increased incidence of megalocytic hepatocytes in the liver (EFSA, 2018a).

2.3.3. *Ochratoxin A*

Ochratoxin A (OTA) is the most toxic member of the ochratoxins as well as the most studied, and it has been classified by IARC as a group 2B carcinogen (i.e. possible human carcinogen) (IARC, 1993). OTA is rapidly absorbed after ingestion with absorption rate depending on the dose and the animal species, 40% in chickens and 66% in pigs. Following absorption, it reaches the systemic circulation where it is bound to plasma proteins, mainly albumin and other serum macromolecules. In many species, including monkeys and humans, the major route of excretion is renal elimination, whereas in rodents, biliary excretion seems to prevail. OTA is not acutely toxic but upon repeated doses it accumulates in the kidney that is the major target organ of its toxicity (Zepnik et al., 2003; EFSA, 2006). It is nephrotoxic and there is evidence that these effects are associated with oxidative stress. Other OTA effects reported are immunotoxicity, genotoxicity, neurotoxicity, teratogenicity and embryotoxicity in human and animals (Marin et al., 2013).

The risks for consumers associated with the dietary exposure to OTA have been assessed by a number of scientific advisory bodies. In 2008, the Joint FAO/WHO expert Committee on Food Additives (JECFA) confirmed a provisional tolerable weekly intake (PTWI) of 100 ng OTA/kg bw already identified in 1995 and retained in 2002. The PTWI is based on a LOEL of 8 μg/kg bw per day for deterioration of renal function in pigs (FAO/WHO, 2008). In 2006, EFSA derived a **tolerable weekly intake (TWI) of 120 ng/kg bw** based on a LOEL of 8 μg/kg per kg bw for early markers of renal toxicity in pigs (the most sensitive animal species) and applying a composite uncertainty factor of 450 for the uncertainties in the extrapolation of experimental data derived from animals to humans as well as for intra-species variability. Recently, new toxicity studies have become available

since 2006. Therefore, EFSA is preparing an update of the scientific opinion addressing also the potential genotoxicity of OTA. In the upcoming opinion, **a $BMDL_{10}$ of 4.73 μg/kg bw per day based on the increased incidence of microscopic kidney lesions in 3-months study with female pigs was identified as** reference point for the risk characterisation of non-neoplastic effects (Krogh et al., 1974). The CONTAM Panel considered that based on the available toxicity data and taking inter- and intraspecies variations into account, a **MOE of 200** would be sufficient to conclude a low health concern for non-neoplastic effects of OTA.

2.3.4. Deoxynivalenol

Since 2004, EFSA has assessed risk of dietary exposure to DON and has identified reference points for both human and animal risk assessment (EFSA, 2004b, 2013a, 2017c). In **pigs**, DON shows generally high absorption (48–65%), extensive organ distribution and also a rapid renal excretion, partly conjugated to glucuronic acid (Alizadeh et al., 2015). Excretion of DON and metabolites occurred through both urinary and biliary routes, with urinary excretion being the most important route in pigs. DON may cause several adverse effects including lesions in the oesophageal region of the stomach, in the liver, the lung and the kidney and changes in different clinical chemistry parameters (plasma nutrients and plasma enzyme activities). Reduced feed intake and bw gain reduction are the critical effects of DON in pigs. The CONTAM Panel identified the **NOAEL of 0.7 mg DON/kg.** This was based on decreased feed consumption, transient reduction in packed cell volume (PCV), decreased serum calcium and phosphorus, increased relative liver weight in pigs given contaminated oat (0.05–3.50 mg/feed) for 3 months (Bergsjo et al., 1993; EFSA, 2017c).

In **poultry**, EFSA CONTAM Panel identified the **NOAEL of 5.0 mg DON/kg feed** basis of the dose that had no effect in feed intake and body weight in broilers given contaminated wheat (0.9-5 mg/feed) for 7 weeks (Awad et al., 2011; EFSA, 2017c). The NOAEL on DON for pig and chicken were expressed as substance concentration in feed. Thus, to convert a feed concentration into a dose a default factor of 0.05 referred to rats has been used (e.g. 1 mg/kg in feed is equivalent to a dose of 0.05 mg/kg bw per day in rats) accordingly to EFSA guidance 2012 (EFSA, 2012a). An uncertainty factor (UF) of 10 for inter-species variation has also been applied.

In **humans**, the CONTAM Panel selected the results of a 2-year study in female mice addressing chronic toxicity of DON as the most appropriate source of data for dose–response modelling (Iverson et al., 1995). Using the BMD approach a

BMDL$_{05}$ of 0.11 mg DON/kg bw per day was derived as reference point from that study (Iverson et al., 1995; EFSA, 2017c). Thus, the **TDI of 1 μg/kg bw per day** was established using the default uncertainty factor of 100 for inter- and intraspecies variability for the sum of DON, 3-Ac-DON, 15-Ac-DON and DON-3-glucoside.

2.3.5. Zearalenone

ZEN exerts its toxic action by interacting with oestrogen receptors and causing an oestrogenic response in animals, being pigs the most sensitive animal. With regards to **pig and poultry**, ZEN has been first assessed as an undesirable substance in animal feed by EFSA in 2004. However, the CONTAM Panel concluded that NOAELs/LOAELs for ZEN in animals could not be derived since the available data were considered inadequate (EFSA, 2004a). A **NOEL of 10.4 μg/kg bw per day** was established for pig based on the oestrogenic effect in female piglets given Fusarium toxin contaminated maize (1.2 mg ZEN and 8.6 mg DON per kg maize) by the CONTAM Panel in 2011 and retained in 2017 (Döll et al., 2003; EFSA, 2011b, 2017b)**. Poultry** responds to the presence of ZEN in feed only at rather high dietary concentrations and can generally be regarded as resistant. Based on decreased in total number of lymphocytes, **NOAELs of 7,500 μg/kg bw per day** was identified for chickens fed 10, 25, 50, 100, 200, 400, and 800 ppm contaminated diet (Chi et al., 1980; EFSA, 2017b).
With regards to **human** risk assessment, in 2011, a tolerable daily intake (TDI) of 0.25 μg/kg body weight (bw) per day was derived from a **NOAEL of 10.4 μg/kg bw per day for oestrogenic effects in female piglets** and applying an uncertainty factor of 40 (4 for interspecies differences in toxicokinetics and 10 for inter-human variability) (Döll et al., 2003; EFSA, 2011b). In 2016, the CONTAM Panel confirmed this TDI and assessed the appropriateness to set a group health based guidance value for ZEN and modified forms. To account for different oestrogenic potencies in ZEN and modified forms, molar potency factors relative to ZEN (relative potency factors (RPFs)) were calculated and applied to exposure estimates of the respective ZEN metabolites. The CONTAM Panel found it appropriate to set a group human **TDI of 0.25 μg/kg bw per day** expressed as ZEN equivalents for ZEN and its modified forms (EFSA, 2016b).

2.3.6. T2 and HT2 toxins

In 2011, the EFSA CONTAM Panel concluded that T2 induces haematotoxicity and myelotoxicity associated with impairment of haematopoiesis in bone marrow in several species. Reduced antibody response observed in a subchronic study in pigs was assessed as critical effect for human risk assessment at that time and, a TDI of

0.1 μg/kg bw was therefore derived for the sum of T2 and HT2. In 2016, upon reviewing new data, the TDI set for T2 in 2011 was revised using a dose-dependent reduction in total lymphocyte counts in a 90-day rats study with T2 of T2 in rats as a critical endpoint
for establishing a chronic **$BMDL_{10}$ of 3.3 μg/kg bw per day** (Rahman et al., 2014). The CONTAM Panel used this value as a reference point for establishing a chronic HBGV for T2 and HT2, i.e. TDI of 0.02 μg/kg bw per day (EFSA, 2016a).

2.3.7. Summary of reference points from individual mycotoxin risk assessments (EFSA CONTAM panel) and resulting assessment groups (AGs) for combined toxicity assessment

Table 28: Reference Points for humans and animals

Substance	Species	Route	Duration (days)	Reference Point (μg/kg bw day)	Toxicity	Ref
Humans						
AFB1	Rat	oral: feed	735	$BMDL_{10}$ = 0.4	hepatotoxicity	(EFSA, 2019b)
OTA	Pig	oral: feed		$BMDL_{10}$ = 4.73	nephrotoxicity	Personal communication from EFSA
DON	Mouse	oral: feed	730	$BMDL_{05}$ = 110	systemic	(EFSA, 2017c)
FB1	Mouse	oral: feed	182	$BMDL_{10}$ = 100	hepatotoxicity	(EFSA, 2018a)
T2/HT2	Rat	oral: feed		$BMDL_{10}$ = 3.33	hemopoietic	(EFSA, 2017a)
ZEN	Pig	oral: feed	Not reported	NOAEL = 10.4	reproductive	(EFSA, 2011b)
Pigs						
DON	Pig	oral: feed	90	NOAEL =350[(a)]	systemic and hepatotoxicity	(EFSA, 2017c)
FB1	Pig	oral: feed	56 and 140	NOAEL = 40	hepatotoxicity and pulmonary oedema	(EFSA, 2018c)

ZEN	Pig	oral: feed	Not reported	NOAEL = 10.4	reproductive	(EFSA, 2017b)
Poultry						
DON	Chicken	oral: feed	35	NOAEL = 2,500[(a)]	systemic	(EFSA, 2017c)
FB1	Chicken	oral: feed	21	NOAEL = 2,000	hepatotoxicity	(EFSA, 2018c)
ZEN	Chicken	oral: feed	Not reported	NOAEL = 7,500	hemopoietic	(EFSA, 2017b)

[(a)]: from 0.7 mg/kg feed and 5 mg/kg feed in pig and chicken, respectively.

Based on (i) co-occurrence data, (ii) common source of exposure and (iii) hazard considerations, the following assessment groups were identified:

- Composition mixture 1 (occurrence data from EFSA): DON + FB + ZEN in animals and DON + FB + T2/HT2 + ZEN in humans;
- Composition mixture 2 (occurrence data from Literature): DON + FB in humans and animals.

The risk assessments for AFs and OTA were performed separately for humans and animals because of both mycotoxins are classified as genotoxic and carcinogenic. With regard to animal risk assessment, the compounds were grouped by NOAEL to be conservative since they could not be grouped by target organ or MoA or mechanisms of action due to lack of data.

provide summary hazard data for each mycotoxin assessment group set in humans and animals.

Table 29, Table 30 and Table 31 provide summary hazard data for each mycotoxin assessment group set in humans and animals.

Table 29: Equivalent factors (EFs) for **human** risk assessment

Mycotoxin	**RP**	**Value (µg/kg bw per day)**	**EF**
Group 1			
DON	$BMDL_{05}$	110	0,030
FB1	$BMDL_{10}$	100	0,033
T-2/HT2	$BMDL_{10}$	3,33	1
ZEN	NOEL	10,4	0,320

Group 2			
DON	$BMDL_{05}$	110	0,909
FB1	$BMDL_{10}$	100	1

Table 30: Equivalent factors (EFs) for **pig** risk assessment

Mycotoxin	**RP**	**Value (µg/kg bw per day)**	**EF**
Group 1			
DON	NOAEL	350	0,030
FB1	NOAEL	40	0,260
ZEN	NOAEL	10,4	1
Group 2			
DON	NOAEL	350	0,114
FB1	NOAEL	40	1

Table 31: Equivalent factors (EFs) for **poultry** risk assessment

Mycotoxin	**RP**	**Value (µg/kg bw per day)**	**EF**
Group 1			
DON	NOAEL	2500	0,800
FB1	NOAEL	2000	1
ZEN	NOAEL	7500	0,267
Group 2			
DON	NOAEL	2500	0,800
FB1	NOAEL	2000	1

2.3.8. Toxicokinetics of single and multiple mycotoxins in different animal species

In the remit of the MYCHIF project, toxicity and toxicokinetic (TK) studies have been retrieved from the public Literature. The collection and the subsequent analysis of TK data *in vivo* allowed writing a review covering the analysis and comparison of kinetic aspects of mycotoxins, alone or in combination, in different animal species (Gkrillas et al., submitted). In brief, the review highlighted the

complexity of studying the TK of mycotoxin mixtures, which needs to be addressed in a case by case scenario. An extensive literature search was performed giving an insight on the currently available data (4057 papers screened) relevant to the hazard assessment of mycotoxins and mycotoxin mixtures by addressing their TK parameters in different animal species. The richest datasets and most important from an agroeconomic point of view are pigs and chickens. Comparison on the sensitivity of chickens and pigs in respect to rats and calculation of uncertainty factors on interspecies toxicokinetic variability was performed. The TK data assessed were: elimination half-life (T½), area under the curve (AUC) and maximum concentration (Cmax) of pigs and chickens in the mycotoxins DON, AFB1, FB1, ZEN and OTA with the respective kinetic parameters of rats. Additionally, the main challenges in the hazard assessment of multiple mycotoxins are reported and the toxicokinetic data needed to perform a more reliable hazard assessment of the co-occurring mycotoxins are discussed.

Mycotoxin dosage, exposure pathway, interspecies and intraspecies differences were identified among the most important parameters that may influence the toxicokinetic of mixtures. As a general remark, a limited availability of scientific papers on mixtures in comparison with the single compounds was reported. Since testing of all mycotoxin mixture combinations is unfeasible, focus should be on the prioritisation of mycotoxin mixtures, creation of harmonised methods for generating *in vitro* and *in vivo* TK data and finally making use of predictive kinetic modelling that include uncertainty and inter and intraspecies variability analysis. All the above will assist in reducing the overall uncertainty and the production of a more robust risk assessment of chemical mixtures for animals and humans.

3. RISK CHARACTERISATION

3.1. Aflatoxins

The $BMDL_{10}$ of 0.4 μg/kg bw per day for the induction of HCC by AFB1 in male rats has been used as a reference point for the risk characterisation of total aflatoxins (AFs) in maize. For substances that are both genotoxic and carcinogenic, the EFSA Scientific Committee stated that a MOE of 10,000 or higher, if based on the $BMDL_{10}$ from an animal carcinogenicity study, would be of low concern from a public health point of view (EFSA, 2005a).

3.1.1. Animals

Table 32: Margin of exposure (MOE) values to the sum of AFs across animal groups in Literature and EFSA data

	LB/UB	MOE to AFs (µg/kg bw per day)							
		Literature				EFSA			
		Max LOD/LOQ		Mean LOD/LOQ		Max LOD/LOQ		Mean LOD/LOQ	
		Estimated exposure	MOE	Estimated exposure	MOE	Estimated exposure	MOE	Estimated exposure	MOE
Piglets	LB	0.008	48.4	0.010	40.4	0.013	30.9	0.032	12.5
	UB	0.449	0.891	0.068	5.9	0.474	0.845	0.104	3.8
Fattening pigs	LB	0.007	58.0	0.008	48.5	0.007	55.1	0.028	14.2
	UB	0.374	1.1	0.057	7.1	0.378	1.1	0.084	4.7
Fattening chickens	LB	0.015	26.9	0.018	22.5	0.016	25.6	0.061	6.6
	UB	0.806	0.496	0.122	3.3	0.814	0.492	0.182	2.2
Laying hens	LB	0.010	40.0	0.012	33.5	0.010	38.1	0.041	9.9
	UB	0.541	0.740	0.082	4.9	0.546	0.733	0.122	3.3

3.1.2. Humans

Comparison of the chronic dietary exposures to AFs to the $BMDL_{10}$ of 0.4 μg/kg bw per day, resulted in MOE values that ranged from 65,479 to 400 (LB/UB) across age groups and datasets (Table 33). Overall, differences can be observed when comparing LB and UB scenarios: at LB the MOEs are mostly above 10,000 indicating low health concern with either max or mean LOD/LOQ approach. However, for the most conservative scenario (UB) the calculated MOEs were below 10,000, and for which health concern may not be excluded (Table 33). In this context, refinement to the approach may be needed.

Table 33: Margin of exposure (MOE) values based on mean dietary exposure to the sum of AFs for the incidence of HCC across age groups

Age group	LB/UB	MOE to AFs (μg/kg bw per day)							
		Literature				EFSA			
		Max LOD/LOQ		Mean LOD/LOQ		Max LOD/LOQ		Mean LOD/LOQ	
		Estimated exposure	MOE	Estimated exposure	MOE	Estimated exposure	MOE	Estimated exposure	MOE
Adolescents	LB	0.00001	49542	0.00001	31720	0.00002	24655	0.00007	6052
	UB	0.00061	652	0.00010	4160	0.00065	616	0.00017	2377
Adults	LB	0.00001	32182	0.00002	20605	0.00002	16016	0.00010	3931
	UB	0.00094	424	0.00015	2702	0.00100	400	0.00026	1544
Elderly	LB	0.00001	65479	0.00001	41924	0.00001	32587	0.00005	7999
	UB	0.00046	862	0.00007	5498	0.00049	815	0.00013	3142

3.2. *Ochratoxin A*

3.2.1. *Animals*

Exposures metrics (LB/UB mean) for OTA were compared with the available reference point ($BMDL_{10}$ of 4.73 μg OTA/kg bw per day) to derive MOEs as illustrated in Table 34.

Table 34: Mean exposure metrics (lower and upper bounds) and associated margin of exposures (MOE) for OTA across farm animal species

Animal species	**LB/UB**	**MOE to OTA (μg/kg bw per day)**			
		Max LOD/LOQ		**Mean LOD/LOQ**	
		Estimated exposure	**MOE**	**Estimated exposure**	**MOE**
Piglets	LB	0.005	995.0	0.008	575.0
	UB	0.036	130.0	0.024	192.0
Fattening pigs	LB	0.004	1239.0	0.007	691.0
	UB	0.030	158.0	0.020	237.0
Fattening chickens	LB	0.008	591.0	0.015	326.0
	UB	0.065	73.0	0.043	109.0
Laying hens	LB	0.006	826.0	0.010	451.0
	UB	0.044	108.0	0.029	160.0

3.2.2. *Humans*

Comparison of the chronic dietary exposures to OTA to the $BMDL_{10}$ of 4.73 μg/kg bw per day, resulted in MOE values that ranged from 240,066 to 184,143 (LB/UB) across age groups and datasets (Table 35). MOE of > 200 from the calculated exposures to this BMDL was considered as of low concern by the EFSA CONTAM Panel. Thus, a MOE > 200 is interpreted as of low concern whereas MOE < 200 may suggest either the need to refine the risk assessment or a risk management consideration. The comparison of exposures with the $BMDL_{10}$ based on the non-neoplastic endpoint resulted in MOEs that remain far above the threshold of 200 in all consumer groups indicating low concern.

Table 35: Margin of exposure (MOE) based on mean dietary exposure to OTA across age groups

Age group	LB/UB	MOE to OTA (μg/kg bw per day)			
		Max LOD/LOQ		Mean LOD/LOQ	
		Estimated exposure	MOE	Estimated exposure	MOE
Adolescents	LB	0.00001	596349	0.00001	369569
	UB	0.00005	93489	0.00003	139324
Adults	LB	0.00001	387379	0.00002	240066
	UB	0.00008	60729	0.00005	90503
Elderly	LB	0.00001	788188	0.00001	488455
	UB	0.00004	123564	0.00003	184143

3.3. *Multiple mycotoxins*

The component-based approach is presented as an exploratory case study for human and animal health risk assessment of combined exposure to multiple mycotoxins in maize food and feed using exposure and hazard metrics for individual mycotoxins. Table 36 to

Table **45** summarise the outcome of the risk assessment. The individual exposure metrics are reported together with the hazard metrics and these are corrected using equivalent factors (EFs) and combined using the dose addition assumption (Sum (Expi)*(EFi)) to derive a margin of exposure (MOE).

3.3.1. *Animal case study*

3.3.1.1. *FB, DON*

For the interpretation of MOEs obtained for the mycotoxin mixture composed by FB and DON, a value of 1 was considered as the threshold. In fact, there was no need to add UF of 100 for intraspecies uncertanty being all RPs calculated for the corresponding species. The MOEs for poultry resulted always above 1 (range: 9.6-16.5) which does not raise concern; whereas they were always below 1 in pigs (range: 0.442-0.612) for which health concern may not be excluded (Table 36 and Table 37). In this context, refinement to the approach may be needed.

3.3.1.2. *FB, DON, and ZEN*

MOEs values for the mixture DON, FB and ZEN resulted always above 1 in poultry (range: 25.4-68.7). In this context, the combined risk is acceptable. On the contrary, MOEs for pigs were close to one (range: 0.891-2.9) for which health concern may not be excluded (Table 38 and Table 39).

Table 36: Normalised exposure (Expi)*(EFi) and MOE to DON, FB in pigs

	EF	(Expi)*(EFi)		Sum (Expi)*(EFi)		MOE		(Expi)*(EFi)		Sum (Expi)*(EFi)		MOE	
		Max LOD/LOQ						Mean LOD/LOQ					
		LB	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**
		Mean	**Mean**					**Mean**	**Mean**				
Piglets													
FB	1	75.0	86.0	78.0	91.0	0.510	0.442	78.0	82.0	82.0	86.0	0.490	0.468
DON	0.114	4.0	4.0					4.0	4.0				
Fattening pigs													
FB	1	62.0	72.1	65.0	75.0	0.612	0.530	65	68	68.0	71.0	0.587	0.561
DON	0.114	3.0	4.0					3	3				

Table 37: Normalised exposure (Expi)*(EFi) and MOE to DON, FB in poultry

	EF	(Expi)*(EFi)		Sum (Expi)*(EFi)		MOE		(Expi)*(EFi)		Sum (Expi)*(EFi)		MOE	
		Max LOD/LOQ						Mean LOD/LOQ					
		LB	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**
		Mean	**Mean**					**Mean**	**Mean**				
Fattening chickens													
FB	1	134.3	155.0	180.8	207.7	11.1	9.6	139.7	146.5	188.6	196.4	10.6	10.2
DON	0.8	46.5	52.8					48.9	50.0				
Laying hens													
FB	1	90.1	104.0	121.3	139.4	16.5	14.3	93.7	98.3	126.5	131.8	15.8	15.2
DON	0.8	31.2	35.4					32.8	33.5				

Table 38: Normalised exposure (Expi)*(EFi) and MOE values in pigs

	EF	(Expi)*(EFi)		Sum (Expi)*(EFi)		MOE		(Expi)*(EFi)		Sum (Expi)*(EFi)		MOE	
		Max LOD/LOQ						Mean LOD/LOQ					
		LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB
		Mean	Mean					Mean	Mean				
Piglets													
DON	0.030	0.577	0.717	4.4	11.7	2.4	0.891	0.6	0.648	5.1	8.9	2.1	1.2
FB	0.260	2.1	6.1					2.4	5.1				
ZEN	1	1.6	4.9					2.2	3.1				
Fattening pigs													
DON	0.030	0.480	0.598	3.6	9.7	2.9	1.2	0.501	0.5	4.3	7.4	2.4	1.4
FB	0.260	1.7	5.1					1.9	4.3				
ZEN	1	1.4	4.1					1.8	2.6				

Table 39: Normalised exposure (Expi)*(EFi) and MOE values in poultry

	EF	(Expi)*(EFi)		Sum (Expi)*(EFi)		MOE		(Expi)*(EFi)		Sum (Expi)*(EFi)		MOE	
		Max LOD/LOQ						Mean LOD/LOQ					
		LB	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**	**LB**	**UB**
		Mean	**Mean**					**Mean**	**Mean**				
Fattening chickens													
DON	0.800	27.9	34.7	43.4	78.8	46.1	25.4	29.1	31.3	46.4	68.3	43.1	29.3
FB	1	14.7	41.8					15.9	35.5				
ZEN	0.267	0.794	2.3					1.0	1.5				
Laying hens													
DON	0.800	18.7	23.3	29.1	52.9	68.7	37.8	19.5	21.0	31.1	45.8	64.2	43.6
FB	1	9.8	28.5					10.9	23.7				
ZEN	0.267	0.532	1.6					0.695	1.0				

3.3.2. *Human case study*

3.3.2.1. *FB and DON*

MOEs ranged between 1186 and 5733 across age groups. Being FB and DON both not genotoxic and carcinogenic, a MOE ≥ 100 is interpreted as of low concern whereas MOE < 100 may suggest either the need to refine the risk assessment or a risk management consideration. Thus combined risk resulted acceptable in all age classes (Table 40, Table 41 and Table 42).

3.3.2.2. *FB, DON, T2/HT2 and ZEN*

MOEs ranged between 581 and 5674 across age groups where the lowest values were observed in adults. Being FB, DON, T2/HT2 and ZEN both not genotoxic and carcinogenic, a MOE ≥ 100 is interpreted as of low concern whereas MOE < 100 may suggest either the need to refine the risk assessment or a risk management consideration. Thus combined risk resulted acceptable in all age classes (Table 43, Table 44 and Table 45).

Table 40: Normalised exposure (Expi)*(EFi) and MOE values in adolescent

		(Expi)*(EFi)µg/kg bw day		Exposure mix (Sum(Expi)*(EFi))		MOE	
		LB	UB	LB	UB	LB	UB
	EF	Mean	Mean	Mean	Mean	Mean	Mean
Normalised exposure (Expi)*(EFi) and MOE values in adolescent with max LOD/LOQ (µg/kg bw per day)							
FB	1	0.008	0.029	0.023	0.055	4338	1826
DON	0.909	0.015	0.026				
Normalised exposure (Expi)*(EFi) and MOE values in adolescent with mean LOD/LOQ (µg/kg bw per day)							
FB	1	0.009	0.024	0.027	0.044	3703	2250
DON	0.909	0.018	0.021				

Table 41: Normalised exposure (Expi)*(EFi) and MOE values in adults

		(Expi)*(EFi)µg/kg bw day		Exposure mix (Sum(Expi)*(EFi))		MOE	
		LB	UB	LB	UB	LB	UB
	EF	Mean	Mean	Mean	Mean	Mean	Mean
Normalised exposure (Expi)*(EFi) and MOE values in adults with max LOD/LOQ (µg/kg bw per day)							
FB	1	0.012	0.044	0.035	0.084	2818	1186
DON	0.909	0.024	0.040				
Normalised exposure (Expi)*(EFi) and MOE values in adults with mean LOD/LOQ (µg/kg bw per day)							
FB	1	0.014	0.037	0.042	0.068	2405	1461
DON	0.909	0.028	0.032				

Table 42: Normalised exposure (Expi)*(EFi) and MOE values in elderly

		(Expi)*(EFi)µg/kg bw day		Exposure mix (Sum(Expi)*(EFi))		MOE	
		LB	UB	LB	UB	LB	UB
	EF	Mean	Mean	Mean	Mean	Mean	Mean
Normalised exposure (Expi)*(EFi) and MOE values in elderly with max LOD/LOQ (µg/kg bw per day)							
FB	1	0.006	0.022	0.017	0.041	5733	2413
DON	0.909	0.012	0.020				
Normalised exposure (Expi)*(EFi) and MOE values in elderly with mean LOD/LOQ (µg/kg bw per day)							
FB	1	0.007	0.018	0.020	0.034	4894	2973
DON	0.909	0.014	0.016				

Table 43: Normalised exposure (Expi)*(EFi) and MOE values in adolescents

		(Expi)*(EFi)µg/kg bw day		Exposure mix (Sum(Expi)*(EFi))		MOE	
		LB	UB	LB	UB	LB	UB
	EF	Mean	Mean	Mean	Mean	Mean	Mean
Normalised exposure (Expi)*(EFi) and MOE values in adolescents with max LOD/LOQ (µg/kg bw per day)							
DON	0.030	0.00015	0.00036	0.00078	0.00372	4293	895
FB1	0.033	0.00023	0.00090				
T-2/HT2	1	0.00004	0.00077				
ZEN	0.320	0.00035	0.00169				
Normalised exposure (Expi)*(EFi) and MOE values in adolescent with mean LOD/LOQ (µg/kg bw per day)							
DON	0.030	0.00018	0.00026	0.00101	0.00225	3299	1482
FB1	0.033	0.00026	0.00076				
T-2/HT2	1	0.00008	0.00028				
ZEN	0.320	0.00048	0.00095				

Table 44: Normalised exposure (Expi)*(EFi) and MOE values in adults

		(Expi)*(EFi)µg/kg bw day		Exposure mix (Sum(Expi)*(EFi))		MOE	
		LB	UB	LB	UB	LB	UB
	EF	Mean	Mean	Mean	Mean	Mean	Mean
Normalised exposure (Expi)*(EFi) and MOE values in adults with max LOD/LOQ (µg/kg bw per day)							
DON	0.030	0.00023	0.00056	0.00119	0.00573	2788	581
FB1	0.033	0.00036	0.00139				
T-2/HT2	1	0.00007	0.00118				
ZEN	0.320	0.00054	0.00259				

Normalised exposure (Expi)*(EFi) and MOE values in adults with mean LOD/LOQ (µg/kg bw per day)							
DON	0.030	0.00028	0.00040	0.00155	0.00346	2143	963
FB1	0.033	0.00040	0.00117				
T-2/HT2	1	0.00013	0.00043				
ZEN	0.320	0.00075	0.00147				

Table 45: Normalised exposure (Expi)*(EFi) and MOE values in elderly

		(Expi)*(EFi)µg/kg bw day		**Exposure mix (Sum(Expi)*(EFi))**		**MOE**	
		LB	**UB**	**LB**	**UB**	**LB**	**UB**
	EF	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**
Normalised exposure (Expi)*(EFi) and MOE values in elderly with max LOD/LOQ (µg/kg bw per day)							
DON	0.030	0.00011	0.00028	0.00059	0.00282	5674	1142
FB1	0.033	0.00018	0.00068				
T-2/HT2	1	0.00003	0.00058				
ZEN	0.320	0.00027	0.00128				
Normalised exposure (Expi)*(EFi) and MOE values in elderly with mean LOD/LOQ (µg/kg bw per day)							
DON	0.030	0.00014	0.00019	0.00076	0.00170	4361	1959
FB1	0.033	0.00020	0.00058				
T-2/HT2	1	0.00006	0.00021				
ZEN	0.320	0.00037	0.00072				

Table 46: Reporting Table: Human and animal risk assessment of combined exposure to multiple mycotoxins

Problem formulation	Description mixture	Composition: Fully defined. Mixtures of mycotoxins, namely *Fusarium* toxins. Composition mixture 1 (occurrence data from Literature): DON + FB in humans and animals. Composition mixture 2 (occurrence data from EFSA): DON + FB + ZEN in animals and DON + FB + T2/HT2 + ZEN in humans.
	Conceptual model	Exposure to the components of mycotoxin mixture (1 and 2) in humans (adolescents, adults and elderly) and farm animal species (fattening chicken, laying hens, piglets and fattening pigs). Exposure pattern: chronic. Hazard data: reference point (RP) for each mycotoxin.
	Methodology	Component based approach (CBA). Assessment group: set using pattern of co-occurrence and toxicity.
	Analysis plan	Risk assessment in food for human health (i.e. adolescents, adults and elderly) and in feed for farm animal health (i.e. poultry and pigs). Margin of exposure (MOE)
Exposure assessment	Mixture composition	Individual mycotoxins
	Summary occurrence data	Occurrence data in food and feed from Literature (for mixture 1) and from EFSA Warehouse (for mixture 2).
	Summary exposure	Mean and P95 occurrence data in food and feed for each component (CBA) combined with (i) food consumption from Comprehensive European Food Consumption Database in humans, and (ii) estimates of feed intakes by the FEEDAP

		panel in animals corrected to default body weight.
	Assumptions	LB and UB highest 95th percentile chronic exposure (conservative)
	Uncertainties	Maximum exposure used: overestimation of exposure
Hazard identification and hazard characterisation	Mixture composition	CBA. Grouping criteria for assessment group: co-exposure and RPs
	Reference points	Humans: RPs for each mycotoxin as $BMDL_{05}$, $BMDL_{10}$, NOEL from chronic studies in test species (i.e. rat, mouse, pig); Animals: RPs for each mycotoxin as $BMDL_{10}$, NOEL (i.e. rat, mouse, pig, chicken)
	Combined toxicity	Dose addition
	Summary hazard metrics	Individual reference points. Equivalent Factors (EFs) for each mycotoxin using the lowest RP of the mixture.
	Uncertainties	Uncertainties in RPs (NOAEL, $BMDL_{05}$, $BMDL_{10}$) for each component. Use of RPs derived from diverse Mode of Action (MoA)
Risk characterisation	Decision points	Apply margin of exposure (MOE)
	Assumptions	Dose addition
	Summary risk metrics	MOE
	Uncertainties	Humans: Uncertainties in reference points particularly for interspecies extrapolation (rat, pig or mouse to humans)
	Interpretation	MOE >100 does not raise human health concerns MOE >10 does not raise animal health concerns

4. UNCERTAINTIES

A qualitative evaluation of the inherent uncertainties in the risk assessment was performed following the EFSA guidance on uncertainty analysis in scientific assessments (EFSA, 2018b). The following paragraphs present in detail the uncertainties affecting different parts of the risk assessment. It includes a qualitative assessment of whether each source of uncertainty leads to over/underestimation of the resulting risk (Table 47).

Occurrence data: The large proportion of samples with left-censored data for some mycotoxins (values below LOD/LOQ) introduced considerable uncertainties to the overall exposure estimate. As a result, the use of the LB in this opinion tends to underestimate, while UB tends to overestimate the dietary exposure. The use of data substitution methods has been evaluated, from which it was concluded that the degree of censoring has a large impact on the uncertainty of the exposure assessment (EFSA, 2010). When assessing exposure to multiple chemical substances with left-censored data, this uncertainty is further magnified (EFSA PPR Panel, 2012b) (EFSA, 2019a).

No data were available on **modified forms** of some mycotoxins, and therefore, a potential presence of other modified forms was not considered for these mycotoxins. This could have resulted in an underestimation of the exposure.

Uncertainties and limitations related to the use of the EFSA Comprehensive Food **Consumption Database** have already been described elsewhere (EFSA, 2011b).

Feed intake: Because there is insufficient data on species-specific compound feeds in EU both for poultry and pigs, default intake values were used to estimate the exposure. Therefore, we could not apply any adjustment to the animal case study that consider only maize-based diet. Therefore, it is most probably that we overestimated the exposure in animals.

Hazard characterisation: uncertainty related to the use of endpoint-related toxicity data for the same effect from another species. The budget uncertainty for the whole approach should consider the lack of data on potential interaction among the considered mycotoxins (i.e., synergistic/additive or antagonistic effect) in the hazard assessment step and the use of reference points derived from a diverse Mode of Action.

Table 47: Summary of uncertainties

Sources of uncertainty		Direction [(a)]
Occurrence data	Using the substitution method at the lower bound (LB) scenario	-
	Using the substitution method at the upper bound (UB) scenario	+
	Lack of data on modified forms	-
Hazard assessment	Lack of data on potential interaction among the considered mycotoxins (i.e. synergism or antagonism) Use of reference points derived from diverse Mode of Action	+/-
Exposure assessment	Feed intake	+

(a): + = uncertainty with potential to cause overestimation of exposure; - = uncertainty with potential to cause underestimation of exposure

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Appendix A - LOD and LOQ cut-off values (μg/kg)

Table A.1.: LOD and LOQ cut-off max values (μg/kg)

	AFB1		**AFB2**		**AFG1**		**AFG2**				**Ref**
Analytical method	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**			
HPLC-LC	0.24	0.6	0.1	0.2	0.1	0.2	0.1	0.2			Personal communication from EFSA
HPLC-FD	1	25	10	41	10	44	0.21	0.6			
HPLC-ECD	0.1	0.3	0.1	0.2	0.1	0.2	0.1	0.2			
LC-MS/MS	2.5	10	1	4	2.5	10	0.23	0.7			
LC-MS	0.5	1	0.5	1	1	2	1	2			
LC-MS-MS (QqQ)	0.2	0.65	0.2	0.65	0.21	0.68	0.2	0.68			
	DON		**3-Ac-DON**		**15-Ac-DON**		**DON-3G**				(EFSA, 2017c)
	LOD	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**			
GC–MS/MS	-	120	-	57	-	25	-	-			
HPLC-UV or HPLC-FLD	-	380	-	60	-	56	-	-			
LC-MS/MS	-	260	-	46	-	250	-	30			
	ENA		**ENA1**		**ENB**		**ENB1**				(EFSA, 2014b)
	LOD	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**			
LC-MS/MS,LC-APCI-MS/MS, LC-ESI-MS/MS	9	17	10	34	12	24	13	26			
	FB1		**FB2**		**FB3**						(EFSA, 2018c)
	LOD	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**					
Methods based on mass spectrometry, spectroscopic detection and gas-chromatographic [(a)]	300	1000	300	1000	50	100					

	NIV										(EFSA, 2013d)
	LOD	**LOQ**									
LC-MS/MS,LC-APCI-MS/MS, LC-ESI-MS/MS	300	620									
	OTA										Personal communication from EFSA
	LOD	**LOQ**									
HPLC-LC	0.28	0.56									
HPLC-UV	-	0.2									
HPLC-FD	0.6	1.3									
HPLC-ECD	0.2	0.6									
LC-MS	1	2									
LC-MS/MS	0.05	0.3									
LC-MS-MS (QqQ)	0.13	0.43									
	T2/HT2		**T2**		**HT2**						(EFSA, 2017a)
	LOD	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**					
Methods based on mass spectrometry, spectroscopic detection and gas-chromatographic [a]	5	20(100)[b]	3	10(50)[b]	3	10(50)[b]					
	ZEN		***α*-ZEL**		**β-ZEL**		**ZAN**		***α*-/β-ZAL**		(EFSA, 2017b)
	LOD	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**	
Methods based on mass spectrometry, spectroscopic detection and gas-chromatographic [a]	10	200	10	50	10	10	10	100	10	50	

–: no information available; TBC: to be confirmed

a: Methods based on mass spectrometry: LC-MS/MS, LC-MS, LC-MS quadrupole, HPLC-ESI-MS. Methods based on spectroscopic detection: HPLC-FD, HPLC-UV, HPLC with standard detection methods,HPLC-CF. Gas-chromatographic methods: GC-MS, GC-HRMS, GC-MS-MS b: food(feed)

Appendix B - Fitting distribution feed and food maize from Literature

B.1 - Fitting distribution of occurrence data from Literature in maize-based feed and food

a) **b)**

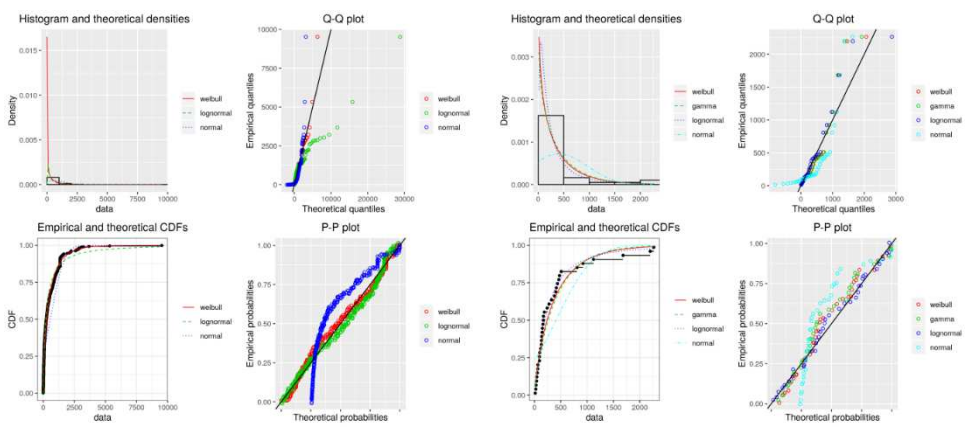

Figure 13 (a, b) : Data and fitting distribution for DON in feed (a) and food (b)

a) **b)**

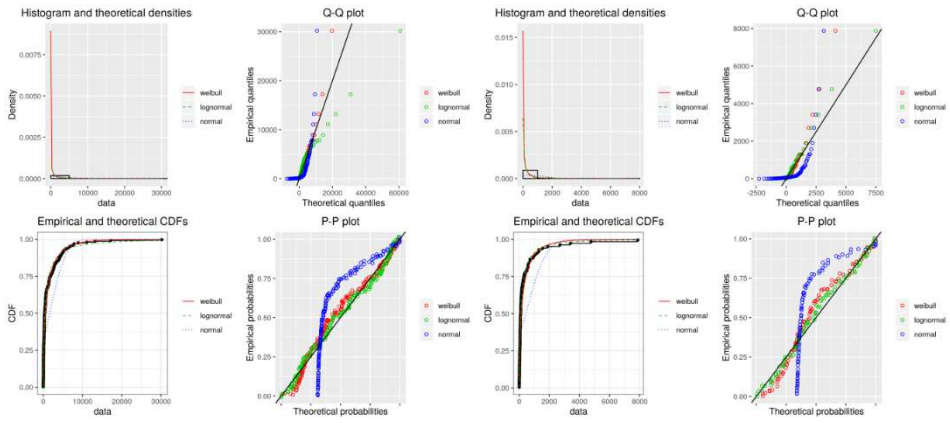

Figure 14 (a, b): Data and fitting distribution for FB in food (a) and feed (b)

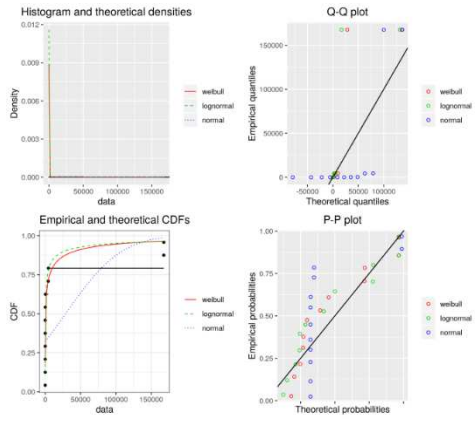

Figure 15: Data and fitting distribution for ENs in feed

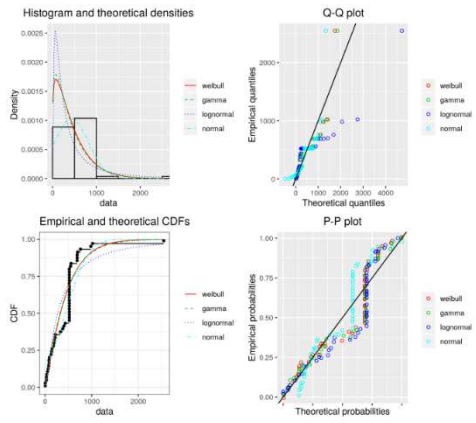

Figure 16: Data and fitting distribution for NIV in feed

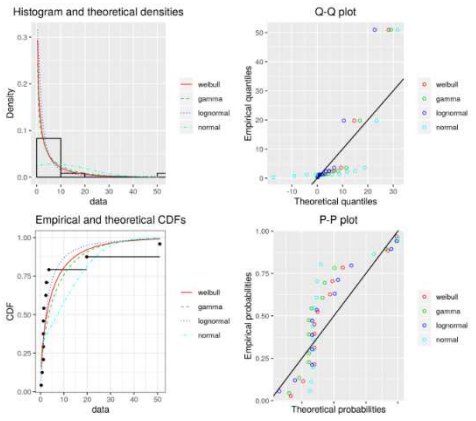

Figure 17: Data and fitting distribution for OTA in feed

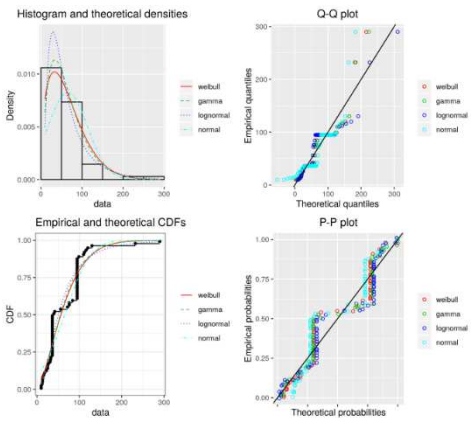

Figure 18: Data and fitting distribution for T2/HT2 in feed

a) **b)**

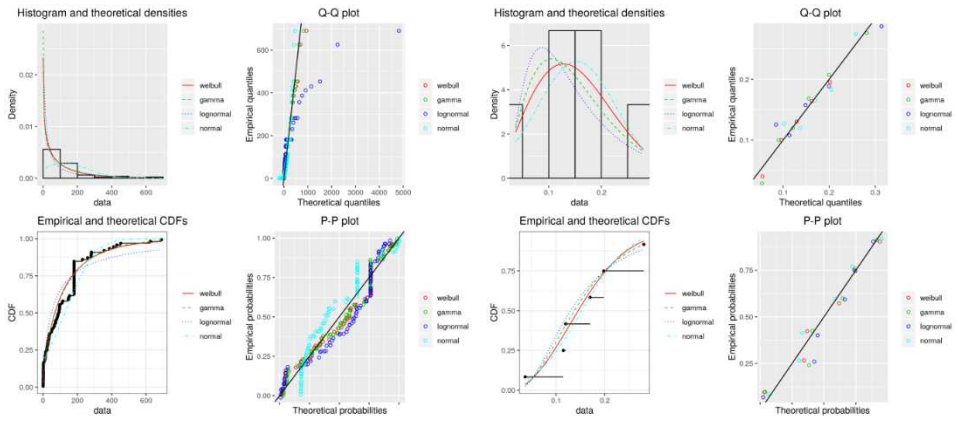

Figure 19 (a, b): Data and fitting distribution for ZEN in feed and food

B.2 - Fitting distribution of occurrence data from EFSA in maize-based feed and food

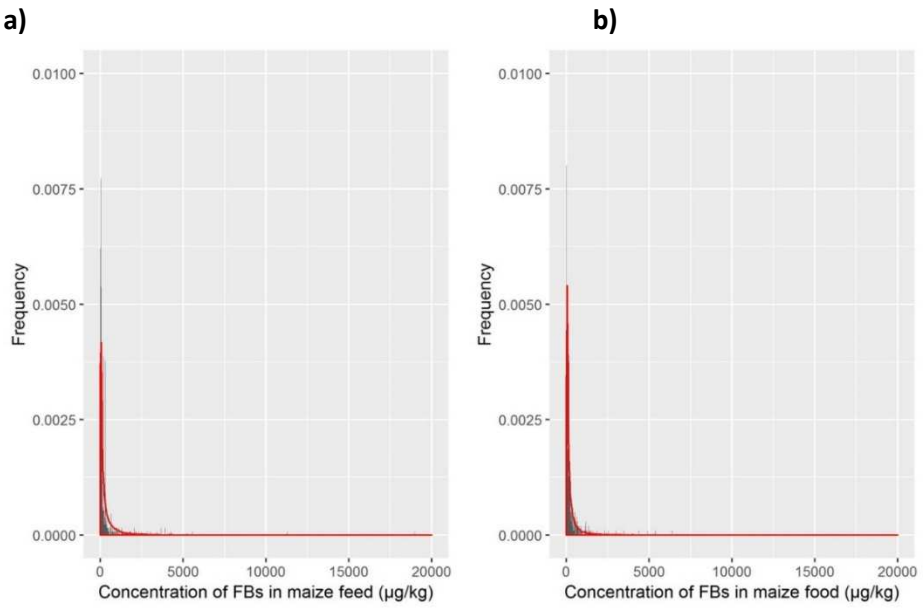

Figure 20 (a, b): Data distribution of FBs in maize feed and food from EFSA database compared to the fitting distribution (red line).

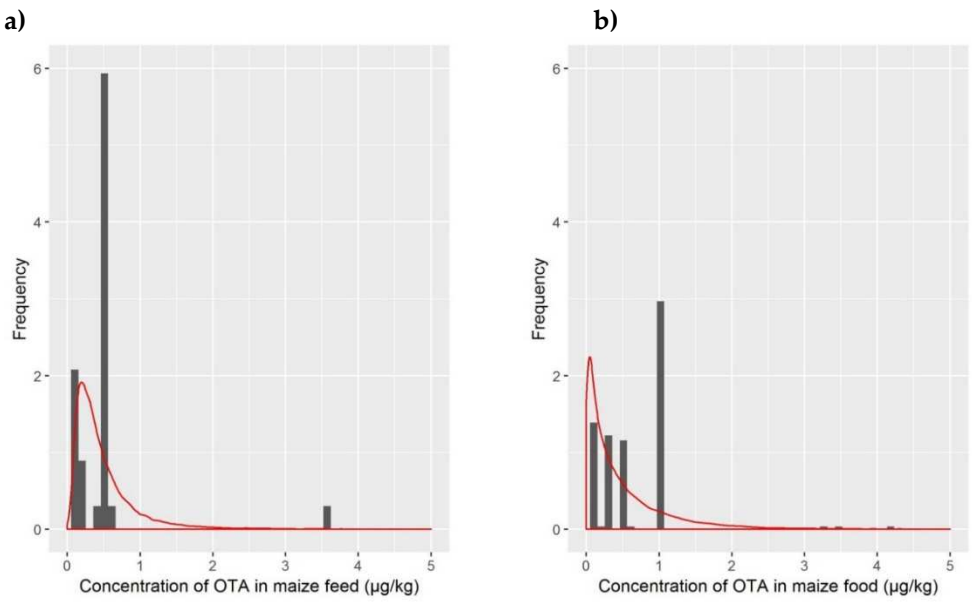

Figure 21 (a,b): Data distribution of OTA in maize feed and food from EFSA database compared to the fitting distribution (red line).

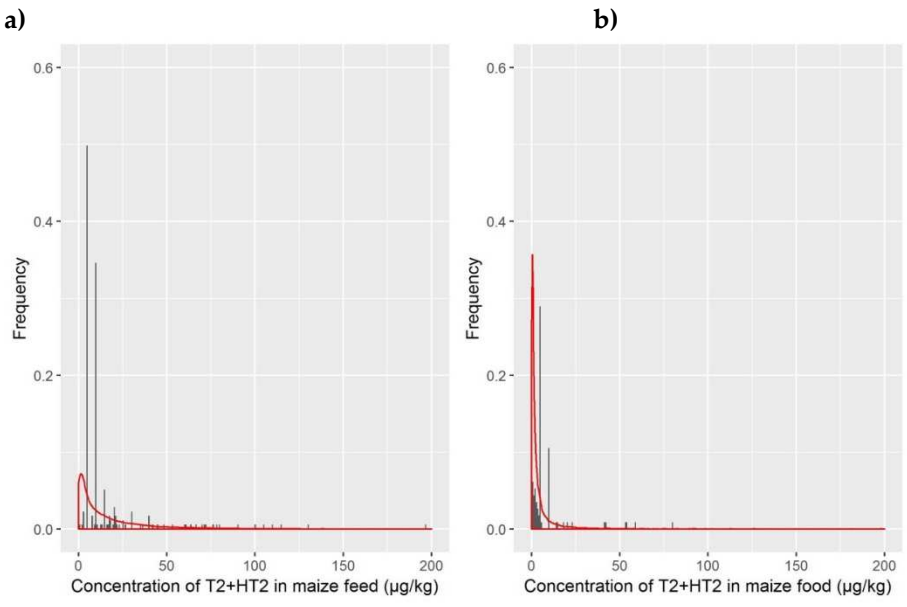

Figure 22 (a, b): Data distribution of T2+HT2 in maize feed and food from EFSA database compared to the fitting distribution (red line).

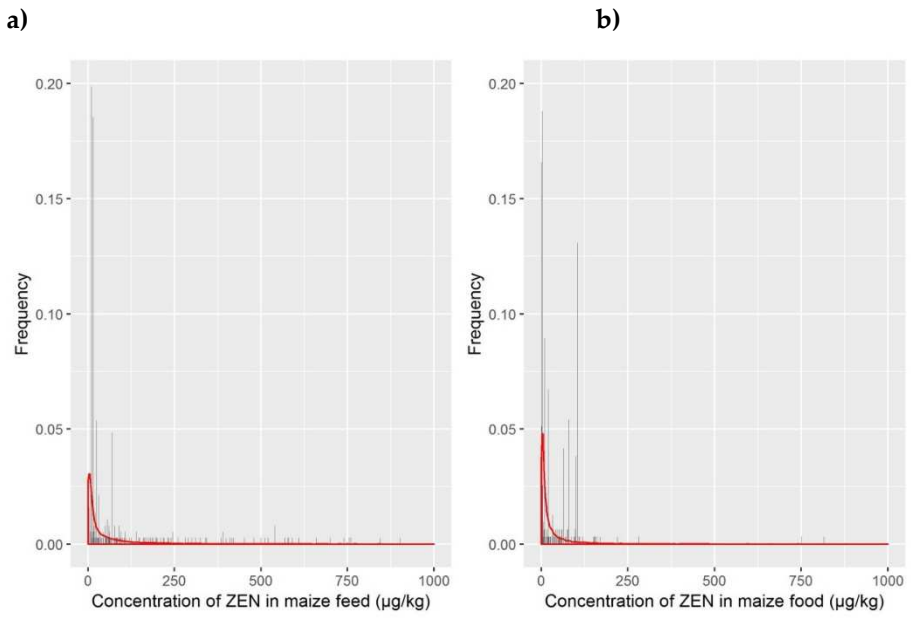

Figure 23 (a, b): Data distribution of ZEN in maize feed and food from EFSA database compared to the fitting distribution (red line)

Final remarks and futures

This study applied a holistic approach to the risk assessment of multiple mycotoxins in food and feed. Thus, variables influencing the production of mycotoxins in the primary production (i.e. environmental variables, ecology of fungi, plant-pathogen interaction and mitigation actions), (co-) occurrence of mycotoxins in food and feed, toxicokinetics (TK) of mycotoxins and their combined toxicity profiles to risk assessment in animals and humans have been investigated to quantify exposure, TK, toxicity for risk characterisation.

From the information collected on environmental, ecological, and agronomic factors that may affect the relative abundance of co-occurring mycotoxins in the contaminated maize, it emerged that profuse literature exists on fungal growth and mycotoxin production, and on factors impacting plant-pathogen interaction. Temperature (T), relative humidity (RH), rainfall (R) and, above all, water activity (a_w) are the most studied ecological factors influencing fungal colonisation of the substrate. Every fungal species has its peculiar ecological needs, and within the same species, optimal conditions for fungal growth do not always correspond to those appropriate to mycotoxin biosynthesis. Main findings on ecological needs (T and a_w range) for fungal growth and mycotoxin production in the most relevant fungal species are available for Aspergilli and Fusaria most frequently isolated in maize. Regarding toxin production, the suitable range of T is always much more limiting compared to growth and they all produce toxins optimally between 25 and 30°C.

Research efforts to support the development of prevention strategies have resulted in developing sound mitigation methods, mainly at pre-harvest stages. Preventive actions in the field have been recognised as an efficient way in reducing the entrance of mycotoxins into the production chain. A major role has been confirmed for the biological control of *A. flavus* to minimize contamination with aflatoxins. Nevertheless, managing mycotoxin contamination requires a comprehensive strategy that includes a correct pre-harvest management and good harvest and post-harvest procedures. Thus, discounting the possibility of mycotoxin contamination in maize cannot yet be foreseen, and further efforts are needed to increase the production of maize with mycotoxins below safe levels set by scientific advisory bodies and introduced in the legislation.

At a global level, climate change is expected to have a significant impact on plant biogeography and fungal populations, with consequences on mycotoxin patterns. Thus, the interest in predictive models for mycotoxin contamination in crops is increasing to account for climate change. Mechanistic models are available for the prediction of AF and FB occurrence in maize crop based on actual weather data, not yet for DON contamination.

In the context of occurrence data, this study has provided a data-based insight from the scientific literature on the (co-) occurrence of mycotoxins (i.e. parent and modified forms) in European core cereals, and has simulated potential patterns of co-exposure by a multinomial model based on observed pattern of co-occurrence. With regard to (co-)occurrence of mycotoxins in core cereals, the results indicate that mycotoxin co-occurrence is common in European cereal-based feed and food, and further highlights the need to conduct monitoring studies for multiple mycotoxins. A large body of evidence collected in this study highlights that maize and wheat may contribute significantly to mycotoxin co-exposure in human and animal species compared to other crops. Cereals and related processed food products are frequently contaminated with mycotoxins, and co-occurrence of Fusarium mycotoxins is highly reported in cereals of major consumption importance in human and animal species, particularly wheat, maize, barley and oat. However, peer-reviewed literature providing information on naturally-co-occurring mycotoxins as well as modified forms in food and feed are still relatively scarce. Still scarce information is available for the co-occurrence of mycotoxins, as well as for the co-occurrence of modified mycotoxins, and more scientific effort is necessary to identify possible combinations of mixtures that can really occur in the real world as well as to better understand the interaction between mycotoxins and its modulation of final toxic effects. Also, the fate of these forms of the toxins during processing is not clearly understood.

Changes in TK parameters and interactions between mycotoxins have been associated with multi-mycotoxin co-exposure. From this data collection exercise, limited *in vitro* and *in vivo* TK and toxicodynamic (TD) data were available from the literature in relation to single and multiple mycotoxins exposure. Indeed, few combined mycotoxin scenarios that can occur in the real world have been tested so far, highlighting the need for a broader assessment of combined TK and toxicity of mycotoxins in test species and farm animals. With respect to TK, the current literature covers mostly pigs and chickens (as relevant species important from an agro-economic point of view) and rats. Mycotoxins type, dosage, exposure pathway, inter- and intra-species differences have been identified as amongst the most relevant parameters that may influence combined TK of mycotoxins.

Finally, the applicability of harmonised risk assessment methodologies has been investigated based on the recent EFSA MIXTOX guidance document to support human and animal health risk assessment of combined exposure to multiple mycotoxins present in maize with non-cancer effects and genotoxic carcinogens, using component-based approaches. In the area of human and animal health, risk assessment of undesirable contaminants, such as mycotoxins, requires identifying unknown modes of action, usually in the absence of kinetic data, for diverse chemicals with rather limited data. For animal health risk assessment differences related to species-specific and interspecies differences in kinetics need to

be taken into account as well as dose-dependent toxicity and methodologies to estimate exposure for risk characterisation. Animal health risk assessment of chemicals aims to protect a range of farm and companion animals from the harmful effects of chemicals present in the feed chain. It also takes into account impacts on human health as an input for exposure assessment via transfer into the food-chain as carry over and residues in animal products (i.e milk, egg and meat).

The use of specific toxic effects for the grouping of chemicals as well as the use of toxicokinetic data and generic physiologically-based kinetic models for farm animal species are options for refining the approaches for the component-based approach proposed by EFSA. In 2014, EFSA recommended to further support quantitative risk assessment through a better understanding of species differences in toxicokinetic (TK) and toxicodynamic (TD) processes for single chemicals and mixtures of chemicals of processes and inter-species differences in such processes and the development of generic biologically-based models (EFSA, 2014). In this respect, physiologically based kinetic (PBK) models are gaining increasing interest as tool for safety in risk assessment of a variety of compounds, including chemicals relevant to food and feed safety. These models allow one to quantitatively link external dose and internal dose for risk assessment of chemicals in an organism without the need to conduct *in vivo* experiments, and to predict concentration-time profiles indicating possible adverse effects and chemical residues in tissues. Recently, three generic multi-compartment physiologically based kinetic (PBK) models have been developed and validated for farm animal species, i.e. cattle, sheep, and swine using a range of compounds mostly eliminated via renal excretion (Lautz et al., 2020).

Regarding the future of risk assessment of multiple mycotoxin, new methods and tools as well as the integration of data are needed to bring a systems toxicology perspective to risk assessment using case studies. In particular, the refinement of assessment factors to determine safe levels of exposure is needed, and the following is recommended:

i. Management of maize genetic resistance, with a particular focus on joint effectiveness towards all mycotoxin producing fungi; further understanding of plant-pathogen interactions and plant defence mechanism, including the role of mycotoxins in maize-fungi cross-talk; extension of biocontrol to Fusaria and pest control as a sustainable approach for mycotoxin mitigation; improvement of the performance of predictive models including investigating the impact of cropping system and of co-occurring fungi on model predictions; prediction of future scenarios of mycotoxin occurrence as a supporting tool for decision makers; further development of alternative biological tools to be applied post-harvest, to improve safe storage or detoxification of contaminated grain to complete the sustainable management of maize value chain;

ii. The creation of harmonised methods for generating (co-)occurrence data is strongly suggested to provide a consistent and coherent background of data for modelling from which prioritisation criteria of mycotoxin mixtures to be tested may be derived. The necessity of continuous monitoring of the major mycotoxins in different agricultural commodities and the creation of harmonised methods for generating accurate (co)occurrence data is strongly suggested. This is mandatory to provide consistent and coherent data for mycotoxin co-occurrence and will allow risk modelling to prioritise key congeners of human and animal health relevance;

iii. TK of mycotoxin mixtures is a complex subject and it can be studied in a case by case scenario. Due to the limited availability of scientific papers on mixtures in comparison with the single compounds there are limited available *in vitro* and *in vivo* data for concurrent mycotoxin exposure. Since testing of all mycotoxin mixture combinations is unfeasible, focus should be on the prioritisation of mycotoxin mixtures, creation of harmonised methods for generating *in vitro* and if necessary *in vivo* TK data;

iv. the use of predictive TK and TD modelling including uncertainty and inter- and intra-species variability analysis should be considered in the future to refine risk characterisation and to assist in reducing the overall uncertainty. The use of structure-based TD models should be considered to understand the mechanisms of toxicity of mycotoxins and to provide a reasonable foothold to develop prioritization criteria.

All the above will assist in reducing the overall uncertainty and the production of a more robust risk assessment of multiple mycotoxins in humans and animals.

List of publications

List of scientific articles that have been prepared in the remit of MYCHIF project and that contributed to the preparation of the thesis.

I. **Palumbo R**, Gonçalves A, Gkrillas A, Logrieco A, Dorne JL, Dall'Asta C, Venâncio A and Battilani P, 2020. Mycotoxins in maize: mitigation actions with a chain management approach. Phytopatologia Mediterranea, 59, *paper published.*

II. Gonçalves A, Gkrillas A, Dorne JL, Dall'Asta C, **Palumbo R**, Lima N, Battilani P, Venâncio A and Giorni P, 2019. Pre- and postharvest strategies to minimize mycotoxin contamination in the rice food chain. Comprehensive Reviews in Food Science and Food Safety, 18:441-454. doi:10.1111/1541-4337.12420, *paper published.*

III. Gonçalves A, **Palumbo R**, Gkrillas A, Dall'Asta C, Dorne JL, Battilani P and Venâncio A, 2019. The route of mycotoxins in the grape food chain. American Journal of Enology and Viticulture, doi:10.5344/ajev.2019.19039, *paper published ahead of print December 16, 2019.*

IV. **Palumbo, R.**; Crisci, A.; Venâncio, A.; Cortiñas Abrahantes, J.; Dorne, J.L.; Battilani, P.; Toscano, P, 2020. Occurrence and co-occurrence of mycotoxins in cereals-based feed and food. Microorganisms, Special Issue "The Route of Mycotoxins from Farm to Fork", 8:74. doi:10.3390/microorganisms8010074, *paper published.*

V. Gkrillas A, Terciolo C, Neves M, **Palumbo R**, Dorne JL, Battilani P, Oswald IP and Dall'Asta C, Comparative Toxicokinetics of mycotoxins, alone or in combination in different animal species: a review. Food and Chemical Toxicology, *paper accepted.*

VI. **Palumbo, R.**; Dorne, J.L.; Battilani, P. Component-based approach for the risk assessment of multiple mycotoxins: a maize case study, *paper in preparation.*

VII. Battilani P, Palumbo R, Giorni P, Dall'Asta C, Dellafiora L, Gkrillas A, Toscano P, Crisci A, Brera C, De Santis B, Cammarano R, Della Seta M, Campbell K, Elliot C, Venancio A, Lima N, Gonçalves A, Terciolo C, Oswald I, 2020. Mycotoxin mixtures in food and feed: holistic, innovative, flexible risk assessment modelling approach: MYCHIF. EFSA supporting publication 2020: 17(1): EN-1757. 161 pp. doi:10.2903/sp.efsa.2020.EN-1757.

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