EXTERNAL SCIENTIFIC REPORT

Mycotoxin mixtures in food and feed: holistic, innovative, flexible risk assessment modelling approach:

MYCHIF

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Abstract

Mycotoxins are toxic compounds mainly produced by fungi of the genera *Aspergillus*, *Penicillium* and *Fusarium*. They are present, often as mixtures, in many feed and food commodities including cereals, fruits and vegetables. Their ubiquitous presence represents a major challenge to the health and well being of humans and animals. Hundreds of compounds are listed as possible mycotoxins occurring in raw and processed materials destined for human food and animal feed. In this study, mycotoxins of major toxicological relevance to humans and target animal species were investigated in a range of crops of interest (and their derived products). Extensive Literature Searches (ELSs) were undertaken for data collection on: (i) ecology and interaction with host plants of mycotoxin producing fungi, mycotoxin production, recent developments in mitigation actions of mycotoxins in crop chains (maize, small grains, rice, sorghum, grapes, spices and nuts), (ii) analytical methods for native, modified and co-occurring mycotoxins (iii) toxicity, toxicokinetics, toxicodynamics and biomarkers relevant to humans and animals (poultry, suidae (pig, wild boar), bovidae (sheep, goat, cow, buffalo), rodents (rats, mice) and others (horses, dogs), (iv) modelling approaches and key reference values for exposure, hazard and risk modelling. Comprehensive databases were created using EFSA templates and were stored in the MYCHIF platform. A range of approaches were implemented to explore the modelling of external and internal exposure as well as dose-response of mycotoxins in chicken and pigs. *In vitro* toxicokinetic and *in vivo* toxicity databases were exploited, both for single compounds and mixtures. However, large data gaps were identified particularly with regards to absence of common statistical and study designs within the literature and constitute an obstacle for the harmonisation of internal exposure and dose-response modelling. Finally, risk characterisation was also performed for humans as well as for two animal species (i.e. pigs and chicken) using available tools for the modelling of internal dose and a component-based approach for selected mycotoxins mixtures.

Summary

Mycotoxins are toxic secondary metabolites produced by fungi. They are present, often as mixtures, in many feed and food commodities including cereals, fruits and vegetables. The most relevant mycotoxins worldwide reported are synthesized by fungal species belonging to three genera: *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp., and to a minor extent by *Alternaria* spp. and *Claviceps* spp. Mycotoxigenic fungi are commonly not host specific, but their occurrence is mainly associated with a specific crop depending on its growing area and the meteorological conditions. A fungus can produce different mycotoxins thus, there can be concurrent contamination of food and feedstuff with multiple mycotoxins. Additionally and adding to the complexity, several fungi can, in quick succession, contaminate food and feed commodities. The native forms of mycotoxins can undergo modifications, as a result of fungi-host plant interaction or during raw product processing; therefore, modified mycotoxins can also occur in addition to the native varieties. Consequently, humans and animals are frequently exposed to more than one mycotoxin simultaneously.

Extensive Literature Searches (ELS) have been performed giving an insight on the currently available data relevant for (i) ecology and interaction with host plants of mycotoxin producing fungi, mycotoxin production, recent developments in mitigation actions of mycotoxins in crop chains (maize, small grains, rice, sorghum, grapes, spices and nuts), (ii) analytical methods for native, modified and co-occurring mycotoxins (iii) toxicity, toxicokinetic, toxicodynamic and biomarkers (BM) for humans and animals (poultry, suidae (pig, wild boar), bovidae (sheep, goat, cow, buffalo), rodents (rat, mice) and others (horses, dogs)), (iv) modelling approaches and key reference values/statements to develop risk modelling. All selected papers contributed to the preparation of this report. Due to the large amount of data collected, a summary of this information has been included in the report while detailed information has been organised in different annexes and scientific publications.

Chapter 2 presents the applied methodology, including review questions and eligibility criteria for study selection (Section 2.1), ELS strategy (Section 2.2) and study selection process (Section 2.3). Section 2.4 described the data extraction process on occurrence and co-occurrence, toxicity and toxicokinetic as well as biomarkers. Section 2.5 presents the development of a structured database across the topics faced in this study. All data extracted for mycotoxin occurrence/co-occurrence, toxicity, toxicokinetics *in vitro,* toxicokinetic *in vivo* and biomarkers have been stored in the MYCHIF platform and EFSA knowledge junction (10.5281/zenodo.3615174).

Temperature (T), relative humidity (RH), rainfall (R) and, above all, water activity (a_w) are the most important ecological factors influencing fungal colonisation of the substrate, and every species has its peculiar ecological needs. Annex A provides a detailed description of the infection cycle and the ecological conditions that influence fungal activity. Fungal infection and subsequent production of mycotoxins begins in the field during plant growth and may continue through harvesting, storage, and processing if ecological conditions stay suitable for fungal activity. Mycotoxins are very stable compounds and accumulate over time, both during crop growth and post-harvest. Therefore, managing mycotoxin contamination requires a comprehensive strategy that includes the correct pre-harvest management and good harvest and post-harvest strategies. Processing will contribute to a reduction in the final mycotoxin content, but the relative reduction is very dependent on the type and sanitary conditions of the raw material, as well as on the technology and operating conditions employed whilst processing is undertaken. Dedicated annexes are presented on the infection cycle, ecology, plant-pathogen interaction of relevant fungi species in the specific host-plant and the state of the art regarding the crop chain management to mitigate the occurrence of mycotoxins in small grains, sorghum, nuts and spices (Annex B – E). In addition, three review papers, respectively on maize (Palumbo et al., 2020b), rice (Gonçalves et al., 2019) and grapes (Gonçalves et al., 2020) were prepared in the remit of MYCHIF project.

The methods of analysis for the determination of mycotoxins are discussed in section 3.3. The list starts with relatively inexpensive rapid screening tools, for both lab and field-based testing, and includes the state of the art of laboratory based screening and confirmatory analytical instruments. Each method has a range of advantages and disadvantages relative to its suitability for single and multiple mycotoxin analysis. However,

when considering methodologies relative to their place of use, there is no one method superior across all envisaged sites of analysis. Combining approaches for field and laboratory-based settings remains an effective monitoring approach in immunological screening and physio-chemical analytical confirmation, though monitoring at the screening level can be limited to a single contaminant. Increasingly with the awareness of multiple mycotoxin contamination, enhanced screening tools are still required for field-based testing for the detection of more than one mycotoxin or mycotoxin families to ensure regulatory compliance. Quite a number of high or ultra-performance chromatography systems coupled to mass spectrometry have now been reported for their use in the determination of the co-occurrence of multiple mycotoxins in different feed and food commodities. However, the challenge still arises to the meaningful interpretation of toxin mixture data to their effects to human and animal health.

Toxicity, toxicokinetics, and toxicodynamic parameters for humans and animals have been collected from papers obtained from ELS, and curated to build three databases: data of toxicokinetic *in vitro,* data of toxicokinetic *in vivo*, and *in vivo* toxicity data. A limited availability of scientific papers on mixtures in comparison with the single compounds was described. Mycotoxins dosage, exposure pathway, interspecies and intraspecies differences were identified among the most important parameters that may influence the toxicokinetics of mixtures. One review paper on toxicokinetic data *in vivo* for different animal species (Gkrillas et al., submitted), was prepared as part of the MYCHIF project. In addition, one original paper was submitted and accepted for publication, on the design of a computational-based driven structure-based toxicodynamic study, comparing the estrogenicity of ZEN, its metabolites and the emerging mycotoxin alternariol (AOH) in human and trout (Dellafiora et al., 2020).

Biomarkers, as a measure of the extent of the exposure to a toxic substance assessed and quantified in body fluids, as a parent molecule or as a metabolite, are examined either in animals (section 3.6.1 and 3.6.2) and in humans (section 3.6.2). For animal species, the review aimed to collect all BM studies assessing markers of exposure in animal species including carryover and residues as well as markers of effects reflecting toxicokinetics and toxicodynamics of mycotoxins (inclusion criteria). Five groups of animal species were categorised and clustered (poultry, bovidae, suidae, rat, mice, and other species). In a similar manner, the relevant literature on human was also reviewed for BM of exposure and effects with a particular interest on available correlations between mycotoxin exposure and health effects. Inclusion criteria selected BM studies for parent mycotoxins or their metabolites, particularly for relevant measurements on multiple mycotoxins. For BM studies, summary tables report measured BM, analytical methods, sampling strategies and summary statistics, including correlation with food intake or exposure assessment.

Two animal case studies were selected and discussed, focussing on pigs and chicken, supported by a reasonable amount of information. For both species, dose-response modelling was performed for mycotoxin mixtures using a component-based approach (CBA) as well as a benchmark modelling approach, as described in section 3.8.1. However, toxicological data concerning mycotoxin mixtures in pigs and chicken were still unsuitable for modelling in terms of toxicokinetic and toxicodynamic parameters, mainly due to poor experimental design.

The human case study (HCS) has been discussed in section 3.8.2. Two different approaches were investigated in the HCS, namely (i) the CBA and (ii) the provisional daily intake (PDI) for two selected mixtures of mycotoxins, which can occur and co-occur in cereal based food products. The description of RA scenarios in hierarchy maps was possible for the HCS, considering the exposure to a defined mixture (i.e., Mixture-2, T2/HT2, DON and NIV) for adults, medium Lower Bound (MB) contamination and mean consumption values. The maps were defined for each mycotoxin of the mixture. These maps represent a useful picture in which it is immediately noticed where the highest ranks in the visual hierarchy are placed.

Specific data gaps, common weaknesses in experimental design and future needs were highlighted. In the context of (co-) occurrence of mycotoxins, 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. Available analytical methods are, in general, satisfactory for the evaluation of the occurrence of mycotoxin mixtures, especially those based on high resolution mass spectrometry (HRMS). Yet, analytical methods are still a limiting

factor for a routinary monitoring of modified mycotoxins in food and feed, both for the cost and the lack of suitable protocols. In the context of toxicity, it has been highlighted the lack of studies dealing with combined toxicity of multiple mycotoxins; further, the harmonisation of methodologies and consensus guidelines for generating *in vitro* and *in vivo* TK and is strongly suggested to provide consistent data for modelling of mycotoxin mixtures.

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1.   Introduction

1.1.   Background and Terms of Reference as provided by the requestor

This contract/grant was awarded by EFSA to:

Contractor/Beneficiary: Università Cattolica del Sacro Cuore

Contract/Grant title: Integrated methodologies for the risk assessment of mycotoxin mixtures in food and feed

Contract/Grant number: GP/EFSA/AFSCO/2016/01

Mycotoxins as mixtures are produced mainly by fungi of the genera *Aspergillus*, *Penicillium* and *Fusarium* co-occurring in host crops. They include many food commodities based on cereals, fruits and vegetables. The ubiquitous presence of fungi and mycotoxins represents a challenge for human and animal health, and the environment. Over the last decade, EFSA has been very active in the area of risk assessment of mycotoxins in food and feed. Just to mention the main activities, aimed to 1) summarise existing knowledge and define scientific opinions; 2) use existing knowledge to predict future scenarios; 3) disseminate existing knowledge:

1. Scientific opinions dealing with risk assessment of mycotoxins in food and feed. These have included well characterised mycotoxins (e.g. aflatoxins, T-2 and HT-2 toxins, zearalenone, etc.) and emerging mycotoxins (e.g. alternaria toxins, beauvericin, enniatins, etc.) (EFSA, 2007, 2011a, b, c, 2014b, 2015b; Mo et al., 2015; EFSA, 2017c, b, a, 2018b);

2. A grant investigating "modelling, predicting and mapping the emergence of aflatoxins in cereals in the EU due to climate change" (Battilani et al., 2012);

3. 2 videos prepared in collaboration with FAO and Università Cattolica del Sacro Cuore.

In addition, EFSA has initiated a series of projects dealing with the risk assessment of chemical mixtures in food and feed: review of the frameworks available for the human risk assessment of chemical mixtures, review of modern methods, including toxicokinetics (TK), OMICs and in silico tools, data collection activities, and an EFSA colloquium on the harmonisation of methods for human and ecological risk assessment of mixtures (EFSA, 2013a, 2014a, 2015a).

From these activities and from the consultation with EFSA Panels and staff dealing with chemical risk assessment and with other experts from international bodies (ECHA, OECD, WHO, etc.), EFSA has formulated several recommendations to further develop methods for the risk assessment for mixtures. These include the refinement of:

1. The detection and reporting of realistic mixtures in food and feed samples for exposure assessment, and

2. The scientific basis to set cumulative assessment groups/assessment groups for chemicals based on their elimination patterns in a number of organisms (TK) and their combined toxicity profiles (dose addition, response addition) or interaction (i.e. synergistic effects/ antagonism) for further refinement of hazard characterization).

3. Combining the refinements of 1 and 2 for risk characterisation and uncertainty analysis based on realistic mixtures in food and feed

These recommendations are directly applicable to the refinement of methodologies for risk assessment of mycotoxin mixtures. In practice, mycotoxin data for specific realistic co-occurrence of free and masked/modified mycotoxins are combined with food consumption patterns for exposure assessment, which

are then further combined with TK/toxicity profiles for risk characterization (De Mattsson, 2007; Steinmetz et al., 2009; Vettorazzi et al., 2013; Zhang et al., 2014; De Boevre et al., 2015).

A major challenge is the understanding of the actual production of mycotoxin mixtures and consequently their realistic occurrence in plants and fruit (Streit et al., 2012). In order to address this challenge, the investigation of complex taxonomic, biochemical, genetic and environmental variables and conditions that would influence the biosynthesis and occurrence of mycotoxin mixtures in plants is needed. These variables include biosynthetic pathways, species/strain specificity on host species, climate change, temperature, resistance of the individual strains to fungicides and associated mechanisms, availability of nutrients and mycotoxin precursors etc., to name but a few (Abou et al., 2013; Blandino et al., 2015; Kabak and Dobson, 2015).

1.2.   Interpretation of the Terms of Reference

The purpose of the contract is to develop an integrated innovative method, supported by modelling, for the risk assessment of mycotoxin mixtures in food and feed, promoting complex system understanding and knowledge gaps identification. Mycotoxins will be used as a model, for the development of a holistic approach, from accumulation during primary production to effects on human and animal health. MYCHIF (**Myc**otoxin mixtures in food and feed: **h**olistic, **i**nnovative, **f**lexible risk assessment modelling approach) method will be applied in case studies, defined in the project and in the future, it will be utilised by risk assessors dealing with mycotoxin mixtures for the food and feed area. The MYCHIF methods/tools development and validation will be described in detail to simplify any enlargement of their use to other contaminants or any integration with new data, if and when requested. The approach will focus on making the best use of the available scientific data, both for risk assessment and mitigation suggestions, and will underline the lack of knowledge as a driver to generate new data, all in a context of sustainability.

The specific objectives are the following:

Objective 1: Review knowledge available on relevant mycotoxin producing fungi in the main crops, accounting for fungi, host crops, environment and their interaction along the crop production chain;

Objective 2: Generate a comprehensive database on occurrence/co-occurrence of mycotoxins in the considered crops;

Objective 3: Review all toxicity data for single and co-occurring mycotoxins;

Objective 4: Generate a comprehensive database on toxicokinetic, toxicodynamic, biomarkers and toxicity data;

Objective 5: Develop a flexible and open access risk assessment model;

Objective 6: Apply the risk assessment model to 5 case studies focused on the maize value chain (cropping system, food and feed, animal products, non-compliance, and future scenarios).

2.   Data and Methodologies

Objectives 1-4 (**Section 1.2**) were addressed through Extensive Literature Searches (ELS). The protocol for the ELSs is described in the respective sections of the report including review questions (**Section 2.1**), eligibility criteria (**Section 2.2**) as well as the screening process (**Section 2.3**).

2.1.   Review questions and eligibility criteria for study selection

The main review questions were identified as follows:

- **Question WP1:** fungi and mycotoxin production in the whole crop production chain, including modelling, fungal growth and toxin production. All relevant articles regarding fungi, host crops, mechanism of plant-pathogen interaction, cropping systems and the role of climate change on the biosynthesis of selected mycotoxins has been considered.

- **Question WP2:** hazard identification and characterisation of mycotoxin mixtures (toxicity data)

The following literature databases were searched to address WP1 and WP2:

- MEDLINE®
- CABI: CAB Abstracts®
- FSTA® - Food Science Technology Abstracts
- CAS - Chemical Abstracts Service
- Biological Abstracts
- ExcerptaMedica
- SCI - Science Citation Index

A pilot search was performed in order to check the consistency on the eligibility criteria. After the refinement of such criteria, ELSs were performed for WP1 and WP2.

Due to the high number of publications retrieved from the databases, the searches were limited to papers published between 2010 and2017. Original articles published in languages such as Chinese, Japanese, Russian, or Arabic were excluded. Patents, editorials and letters were excluded from the searches.

Table 1: Eligibility criteria for WP1

STUDY QUESTION WP 1	Fungi and mycotoxin production in the whole crop production chain, including modelling fungal growth and toxin production. All relevant articles regarding fungi, host crops, mechanism of plant-pathogen interaction, cropping systems and the role of climate change in biosynthesis of selected mycotoxins has to be considered	
Study design	In	*In vitro/in vivo* studies
	Out	
Language of the full text	In	Full-text document in English
	Out	Chinese, Japanese, Russian, or Arabic
Time	In	2010-2017(a)
Publication type	In	Report is primary research (i.e. studies generating new data) or data collection or systematic reviews
	Out	Patents, editorials and letters

(a) Additional records preceding 2010 were retrieved from the list of available references

Table 2: Eligibility criteria for WP2

STUDY QUESTION WP 2	Is the study addressing all toxicokinetic data for single and/or co-occurring mycotoxins

Study design	In	*Invitro/in vivo* studies
	Out	
Language of the full text	In	Full-text document in English
	Out	Chinese, Japanese, Russian, or Arabic
Time	In	2010-2017[a]
Publication type	In	Report is primary research (i.e. studies generating new data) or data collection or systematic reviews
	Out	

(a) Additional records preceding 2010 were retrieved from the list of available references

2.2. Extensive Literature Search (ELS)

The strategy for the extensive literature searches was designed according to EFSA guidance document on systematic review (EFSA, 2010a). Here, a strategy between *Recall* (retrieving as many relevant citations as possible) and *Precision* (eliminating as much noise as possible) was applied.

All partners of the consortium were involved in the definition of: i) review questions, ii) search strategy and iii) identification of relevant key words.

2.2.1. WP1: Fungi and mycotoxin production

The search strategy was performed for seven (7) crops/crop groups, namely MAIZE, SMALL GRAINS, RICE, SORGHUM, NUTS, GRAPES and SPICES.

The following crop-related terms were considered and searched within the title, the abstract or the keyword field:

1. **GRAPE#/TI,AB OR GRAPE#/BI**
2. **MAIZE/TI,AB OR CORN/TI,AB OR ZEA MAYS/BI OR MAIZE/BI OR CORN/BI**
3. **RICE/TI,AB OR RICE/BI OR ORYZA SATIVA/TI,AB OR ORYZA SATIVA/BI**
4. **SORGHUM/TI,AB OR SORGHUM/BI**
5. **SMALL GRAIN#/TI,AB**

   BARLEY/TI,AB OR HORDEUM VULGARE/TI,AB

   WHEAT/TI,AB OR TRITICUM VULGARE/BI

   OAT/TI,AB OR AVENA SATIVA/TI,AB

   AVENA/BI

   RYE/TI,AB OR SECALE CEREALE/TI,AB OR SECALE/BI

   EINKORN/TI,AB OR TRITICUM MONOCOCCUM/TI,AB

   SPELT/TI,AB OR TRITICUM SPELTA/TI,AB

   TRITICALE/TI,AB OR TRITICALE/BI

   EMMER/TI,AB OR EMMER/BI
6. **SPICE#/TI,AB OR SPICES/BI**
7. **PISTACHIO#/TI,AB OR PISTACHIO/BI OR PISTACIA VERA/TI,AB OR PEANUT#/TI,AB OR PEANUT/BI OR ARACHIS HYPOGAEA/TI,AB OR ALMOND#/TI,AB OR ALMOND/BI OR AMYGDALUS COMMUNIS/TI,AB OR HAZELNUT#/TI,AB OR**

HAZELNUT/BI OR OIL# SEED#/TI,AB OR NUT# TREE#/TI,AB OR TREE# NUT#/TI,AB

Each group of crop-related terms was connected using the Boolean operator **AND** for the following sets of keywords:

(FUNG##/TI,AB OR FUSARIUM/TI,AB OR ASPERGILLUS/TI,AB OR PENICILLIUM/TI,AB OR CLAVICEPS/TI,AB OR ALTERNARIA/TI,AB OR AAL TB TOXIN/TI,AB OR ALTERNARIOL/TI,AB OR ALTERNARIOL/BI OR FUSARIUM/BI OR FUNGI/BI OR ASPERGILLUS+NT/CT OR PENICILLIUM/BI OR CLAVICEPS/BI OR ALTERNARIA/BI)

Afterwards, these were connected using the **AND** Boolean operator to a set of terms including **all mycotoxin-related terms and synonyms (e.g.: Mycotoxin# OR Aflatoxin# OR Ochratoxin# OR Fuminosin# etc.).**

Each set were subsequently connected using the **AND** Boolean operator for each of the following search-group, namely, cropping, biosynthesis, occurrence and modelling. An example of search strings applied to maize is illustrated in Table 3.

Table 3: Search strings applied to the extensive literature searches on maize mycotoxins (for WP1)

WP1. SEARCH STRINGS IN MAIZE	
Cropping	**[**(maize OR corn OR *Zea mays*) **AND** (fung* OR Fusarium OR Aspergillus OR Penicillium OR Claviceps OR Alternaria OR AAL TB toxin OR Alternariol) **AND** (Mycotoxin* OR toxin* OR Aflatoxin* OR AF* OR Fumonisin OR FB* OR Deoxynivalenol OR DON OR Ochratoxin OR OTA OR Zearalenone OR ZEN OR ZEA OR Patulin OR PAT OR T2 OR HT2 OR Trichothecene* OR ergot alkaloids OR Beauvericin OR BEA OR Enniatin* OR ENN OR moniliformina OR MON) OR mycotoxin*(near)occur ORmycotoxin (near)Cooccur*OR mycotoxin (near)Co-occur* OR mycotoxin (near)Modified OR mycotoxin (near)Masked OR mycotoxin (near)Combined OR mycotoxin (near)Mixture OR mycotoxin (near)Conjugated)**]AND[(**Cropping system OR Harvest OR Pre-harvest OR Irrigation OR Pest control OR Disease control OR Occurrence OR co-occur* OR growth OR sporulation OR ecolog* OR water activity OR Climat* change* OR meteorological(s)change* OR global warming OR weather conditions OR tropical* OR temperature OR climat* variation* OR metereological* variation*) **AND** (Post-harvest OR processing OR products OR storageORDerived products OR Processed products OR Final products) **]**
Biosynthesis	**[**(maize OR corn OR zea mays) **AND** (fung* OR Fusarium OR Aspergillus OR Penicillium OR Claviceps OR Alternaria OR AAL TB toxin OR Alternariol) **AND** (Mycotoxin* OR toxin* OR Aflatoxin* OR AF* OR Fumonisin OR FB* OR Deoxynivalenol OR DON OR Ochratoxin OR OTA OR Zearalenone OR ZEN OR ZEA OR Patulin OR PAT OR T2 OR HT2 OR Trichothecene* OR ergot alkaloids OR Beauvericin OR BEA OR Enniatin* OR ENN OR moniliformina OR MON) OR mycotoxin*(near)occur ORmycotoxin (near)Cooccur*OR mycotoxin (near)Co-occur* OR mycotoxin (near)Modified OR mycotoxin (near)Masked OR mycotoxin (near)Combined OR mycotoxin (near)Mixture OR mycotoxin (near)Conjugated)**]AND[**Biosynthesis**]**
Occurrence	**[**(maize OR corn OR zea mays) **AND** (fung* OR Fusarium OR Aspergillus OR Penicillium OR Claviceps OR Alternaria OR AAL TB toxin OR Alternariol) **AND** (Mycotoxin* OR toxin* OR Aflatoxin* OR AF* OR Fumonisin OR FB* OR

	Deoxynivalenol OR DON OR Ochratoxin OR OTA OR Zearalenone OR ZEN OR ZEA OR Patulin OR PAT OR T2 OR HT2 OR Trichothecene* OR ergot alkaloids OR Beauvericin OR BEA OR Enniatin* OR ENN OR moniliformina OR MON) OR mycotoxin*(near)occur ORmycotoxin (near)Cooccur*OR mycotoxin (near)Co-occur* OR mycotoxin (near)Modified OR mycotoxin (near)Masked OR mycotoxin (near)Combined OR mycotoxin (near)Mixture OR mycotoxin (near)Conjugated)]**AND[** (food OR dairy products OR milk OR yogurt OR yoghurt OR cheese OR breakfast cereal* OR gluten free product* OR bread OR pasta OR wine OR raisin OR current OR juice OR beer OR malt OR feed OR silage OR forage OR fodder OR hay OR concentrate OR snaplage OR earlage OR By-Products OR Co-Products OR meal OR grain OR whole grains OR compliant OR legislation)**]**
Modelling	**[**(maize OR corn OR zea mays) **AND** (fung* OR Fusarium OR Aspergillus OR Penicillium OR Claviceps OR Alternaria OR AAL TB toxin OR Alternariol) **AND** (Mycotoxin* OR toxin* OR Aflatoxin* OR AF* OR Fumonisin OR FB* OR Deoxynivalenol OR DON OR Ochratoxin OR OTA OR Zearalenone OR ZEN OR ZEA OR Patulin OR PAT OR T2 OR HT2 OR Trichothecene* OR ergot alkaloids OR Beauvericin OR BEA OR Enniatin* OR ENN OR moniliformina OR MON) OR mycotoxin*(near)occur ORmycotoxin (near)Cooccur*OR mycotoxin (near)Co-occur* OR mycotoxin (near)Modified OR mycotoxin (near)Masked OR mycotoxin (near)Combined OR mycotoxin (near)Mixture OR mycotoxin (near)Conjugated)**]AND[**Modelling**]**

For each group of crops, four (4) extensive literature searches were performed, for a total of 24 searches. From the total number of records, duplicate records from different database were removed. The number of selected records was 18774, as summarized in Table 4.

Ad-hoc searches were made for specific topics leading up to 2000 when available information was insufficient.

Table 4: Summary data regarding ELS for WP1

EFSA SYSTEMATIC REVIEW	**Extensive literaturesearch as preparatory work for:** **Fungi and mycotoxin production**
STUDY QUESTION	Fungi and mycotoxin production in the whole crop production chain, including modelling fungal growth and toxin production. All relevant articles regarding fungi, host crops, mechanism of plant-pathogen interaction, cropping systems and the role of climate change in biosynthesis of selected mycotoxins has to be considered
Date access	April 2017
Total N° of records retrieved	18774

The references downloaded from literature databases, including all indexed fields per hit (e.g. title, authors, abstract, etc.), were managed - stored and classified - into separate Endnote ^{TM} 8 files, allowing a count of the individual hits per search and duplicate record removal.

2.2.2. WP2: Toxicity data for mycotoxins and hazard identification

For WP2, the ELS provided a basis for toxicity data collection for single and co-occurring mycotoxins.

Due to the high number of documents present in the databases, the search was initially limited to papers published within the time span 2010-2017, as also agreed for WP1. Nonetheless, the paucity of relevant data for toxicity *in vivo* made searching the scientific literature published before 2010 necessary to reach a suitable data set for modelling (see sections below). This additional expert literature search found papers spanning from 1976. Also in this search, original articles published in languages such as Chinese, Japanese, Russian, or Arabic, as well as patents, editorials and letters were excluded from the search.

The core of the ELS for WP2 is represented by the set containing all mycotoxin-related terms, in the title, the abstract or the controlled terms field of the record:

> MYCOTOXIN#/TI,AB OR AFLATOXIN#/TI,AB OR FUMONISIN#/TI,AB OR DEOXYNIVALENOL/TI,AB OR OCHRATOXIN#/TI,AB OR ZEARALENONE/TI,AB OR PATULIN/TI,AB OR TRICHOTHECENE#/TI,AB OR ERGOT ALKALOID#/TI,AB OR T2 TOXIN#/TI,AB OR HT2 TOXIN#/TI,AB OR T2 OR TRIOL/TI,AB OR BEAUVERICIN/TI,AB OR ENNIATIN#/TI,AB OR MONILIFORMIN/TI,AB OR MYCOTOXINS+NT/CT OR AFLATOXINS+NT/CT OR FUMONISINS/BI OR DEOXYNIVALENOL/BI OR OCHRATOXINS/BI OR ZEARALENONE/BI OR PATULIN/BI OR TRICHOTHECENES+NT/CT OR ERGOT ALKALOIDS+NT/CT OR BEAUVERICIN/BI OR ENNIATINS/BI OR MONILIFORMIN/BI OR FUMONISIN/BI OR OCHRATOXIN/BI ORTRICHOTHECENE/BI OR BEAUVERICINS/BI OR ENNIATIN/BI

This set was connected by the **AND** Boolean operator to each of the following aspects:

- **TOXICITY**
- **BIOMARKERS**
- **ANALYTICAL METHODS**
- **TOXICOKYNETICS**
- **MODELLING**

Five (5) literature searches were performed, one for each aspect. Each search was later limited to the following species:

> (HUMANS/BI OR CHILD/BI OR INFANT/BI) OR (MAN OR MEN OR WOMAN OR WOMEN) OR (CHILD OR CHILDREN) OR (FOETUS OR FETUS OR TODDLER#) OR (INFANT# OR INFANCY OR ADOLESCENT#) OR (ADOLESCENCE OR ADULT#) OR ADOLESCENTS/BI OR (RAT OR RATS OR MOUSE OR MICE) OR (PIG# OR SWINE OR COW# OR CATTLE OR CHICKEN) OR (POULTRY OR TURKEY OR DUCK OR FISH##) OR (CAT OR CATS OR DOG OR DOGS) OR (RABBIT OR RABBITS) OR (RATS/BI OR DOGS/BI) OR (CHICKENS/BI OR CATTLE/BI) OR (POULTRY/BI OR TURKEY/BI) OR (DUCKS/BI OR FISHES/BI)

From the total number of records for each of the five subtopics, from different database were removed; the gran-total of selected records was 20440.

Table 5: Summary data regarding ELS for WP2

EFSA SYSTEMATIC REVIEW	EXTENSIVE LITERATURESEARCH AS PREPARATORY WORK FOR: Toxicity data for mycotoxins and hazard identification
STUDY QUESTION	Toxicity data for mycotoxins and hazard identification
Date accessed	May 2017
Total N° of records retrieved	20440

The research strategy and the terminology for the search were agreed upon and adopted in advance by the relevant partners responsible for managing WP2. The keywords used were related to mycotoxins, their toxicity (single and mixture) in human health, livestock and companion animals.

The output of the search was organised in an Endnote ™ 8 file where the total number of papers was counted and duplicates were removed.

2.3. Study selection process

2.3.1. WP1

The resulting records underwent a two-step selection procedure after duplicate removal:

1) **Screening of title and abstract** to identify potentially relevant studies that will be included for full-text screening, applying the eligibility criteria described in section 2.1. If the information contained in the title or abstract was not relevant to the research objectives, the article was not selected for full-text assessment.

2) **Full-text screening.** Subsequent screening for studies passing the first step was based on the full-text article to assess if the article was relevant to the research objectives. Regarding occurrence, the presence of quantitative data was considered as inclusion criteria.

Articles that were excluded during screening were stored in Endnote ™ 8. The outcomes of the screening process are reported in Table 6. Furthermore, the results of the different phases of the study selection process are duly reported in flowcharts by each crop search (Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6 and Figure 7).

The screening process followed the same approach for all considered crops. During the screening process, studies were categorised in different groups corresponding to the WP1 tasks. All selected papers contributed to the preparation of individual reports and review articles.

Table 6: Number of records in each step of the selection flow by each crop search-groups

	Total N° of records	N° of duplicates	Records removed applying criteria (title and abstract)	N° records included after title and abstract screening	N° records remaining after full-text retrieval and screening
Maize					
Cropping	1611	154	942	515	**452**
Modelling	82	1	58	23	**17**
Occurrence	5016	97	4555	364	**222**
Small grains					
Cropping	1311	29	979	303	**241**
Modelling	83	1	50	32	**25**
Occurrence	4449	0	3914	535	**266**
Rice					
Cropping	990	18	850	122	**107**
Modelling	38	2	32	4	**3**
Occurrence	2968	49	2743	176	**63**
Sorghum					

Cropping	161	3	135	23	**23**
Modelling	6	1	5	0	**0**
Occurrence	593	13	537	43	**21**
Nuts					
Cropping	459	21	235	203	**39**
Modelling	13	0	8	5	**3**
Occurrence	1088	20	968	100	**13**
Grapes					
Cropping	502	10	356	136	**106**
Modelling	18	0	14	4	**4**
Occurrence	1453	32	1322	99	**33**
Spices					
Cropping	168	6	136	26	**20**
Modelling	2	0	1	1	**1**
Occurrence	383	14	297	72	**39**

Figure 1: Flow diagram of the study selection in maize

SMALL GRAINS

Figure 2: Flow diagram of the study selection in small grains

RICE

Figure 3: Flow diagram of the study selection in rice

SORGHUM

Cropping and modelling

Occurrence

Identification

| Total number of records (n=167) | → | Duplicates (n=4) |

| Records after duplicates removed (n=163) |

Screening

| Records screened by title and abstracts (n=163) | → | Records excluded (n=140) |

| Records screened by full-text (n=23) | → | Records excluded (n=0) |

Eligibility

| Studies included (n=23) |

Identification

| Total number of records (n=593) | → | Duplicates (n=13) |

| Records after duplicates removed (n=580) |

Screening

| Records screened by title and abstracts (n=580) | → | Records excluded (n=537) |

| Records selected for full-text screening (n=43) | → | Records excluded (n=22) |

| Studies included (n=21) | → | no-EU countries (n=20) |

Eligibility

| Records used for data extraction (n=2) | ← | Integrating search 2000-2009 and 2017-2018 (n=1) |

| Records occurrence (n=1) | | Records co-occurrence (n=1) |

Figure 4: Flow diagram of the study selection in sorghum

NUTS

Cropping and modelling

Occurrence

Identification

| Total number of records (n=472) | → | Duplicates (n=21) |

| Records after duplicates removed (n=451) |

Screening

| Records screened by title and abstracts (n=451) | → | Records excluded (n=243) |

| Records selected for full-text screening (n=208) | → | Records excluded (n=166) |

Eligibility

| Studies included in full-text screening (n=42) |

Identification

| Total number of records (n=1088) | → | Duplicates (n=20) |

| Records after duplicates removed (n=1068) |

Screening

| Records screened by title and abstracts (n=1068) | → | Records excluded (n=968) |

| Records selected for full-text screening (n=100) | → | Records excluded (after full-text retrieval and screening) (n=87) |

| Studies included (n=13) | → | no-EU countries (n=8) |

Eligibility

| Records used for data extraction (n=5) |

| Records occurrence (n=5) | | Records co-occurrence (n=0) |

Figure 5: Flow diagram of the study selection in nuts

GRAPES

Cropping and modelling

Identification

Total number of records (n=520) → Duplicates (n=10)

Records after duplicates removed (n=510)

Screening

Records screened by title and abstracts (n=510) → Records excluded (n=370)

Records selected for full-text screening (n=140) → Records excluded (n=30)

Eligibility

Studies included in full-text screening (n=110)

Occurrence

Identification

Total number of records (n=1453) → Duplicates (n=32)

Records after duplicates removed (n=1421)

Screening

Records screened by title and abstracts (n=1421) → Records excluded (n=1322)

Records selected for full-text screening (n=99) → Records excluded (after full-text retrieval and screening) (n=66)

Studies included (n=33) → no-EU countries (n=14)

Eligibility

Records used for data extraction (n=19)

Records occurrence (n=7)   Records co-occurrence (n=12)

Figure 6: Flow diagram of the study selection in grapes

SPICES

Cropping and modelling

Identification

Total number of records (n=170) → Duplicates (n=6)

Records after duplicates removed (n=164)

Screening

Records screened by title and abstracts (n=164) → Records excluded (n=137)

Records screened by full-text (n=27) → Records excluded (n=6)

Eligibility

Studies included (n=21)

Occurrence

Identification

Total number of records (n=383) → Duplicates (n=14)

Records after duplicates removed (n=369)

Screening

Records screened by title and abstracts (n=369) → Records excluded (n=297)

Records selected for full-text screening (n=72) → Records excluded (after full-text retrieval and screening) (n=33)

Studies included (n=39) → no-EU countries (n=30)

Eligibility

Records used for data extraction (n=9)

Records occurrence (n=8)   Records co-occurrence (n=1)

Figure 7: Flow diagram of the study selection in spices

The ELS library for **maize** included 6709 records, organized as indicated in Table 6. The screening process selected 452 records accounting for fungi, host crops, environment and their interaction along the crop production chain and 17 regarding modelling. Regarding occurrence, the presence of quantitative data was considered as inclusion criteria. In maize, papers passing the full text screening were 222 (40 with occurrence data from EU countries and 182 with occurrence data from non EU countries). Only the papers with occurrence data from EU countries were extracted (Figure 1). Due to the limited number of papers retrieved on co-occurrence in maize, an additional literature search was performed. The literature search was extended to the timeframe from 2000 to 2009 and 2017-2018 (June). In total an additional number of 12 papers were considered for the extraction of mycotoxin occurrence in maize. More details on the integrating ELS for maize are reported below. The following databases were used:

• MEDLINE®
• CABI: CAB Abstracts®
• PubMed®

The following terms were considered from the title and/or the abstract:

((maize or corn or zea mays) and (fung* or Fusarium or Aspergillus or Penicillium or Claviceps or Alternaria or AAL TB toxin or Alternariol) and (Mycotoxin* or toxin* or Aflatoxin* or AF* or Fumonisin or FB* or Deoxynivalenol or DON or Ochratoxin or OTA or Zearalenone or ZEN or ZEA or Patulin or PAT or T2 or HT2 or Trichothecene* or ergot alkaloids or Beauvericin or BEA or Enniatin* or ENN or moniliformina or MON) and ((occur* or co-occur* or cooccur*))

For **small grains,** records were initially screened for their relevance to WP1, considering the different species included (barley, oat, wheat and other grains). Within the set of data from the small grains library, 285 publications reported the occurrence of mycotoxins in different food commodities and were included. Of these, 151 were from non-EU countries, remaining 149 in which occurrence and co-occurrence data were present (Figure 2). Fifteen additional papers were included from 2000 to 2009.

The ELS library for **rice** included 3996 records, organized as indicated in Table 6. After duplicate removal and first screening, 126 records were selected from cropping and modelling search-groups. The full text of most of these records was retrieved for the report preparation, but, at this stage, some records were excluded (16 records) for different reasons:

• a full text was not available (e.g., abstract published in conference proceeding books);
• not possible to retrieve the full text (e.g., error in doi); or
• other

Based on the information retrieved so far, it is possible to state that most of the information relates to the occurrence of mycotoxins, mainly from fermented rice products, where citrinin is the main occurring mycotoxin. Although 63 records were selected, only 4 were used to extract data on occurrence in EU countries (Figure 3). Four additional papers were included from 2000 to 2009.

The ELS library for **sorghum** included initially 760 records, organized as indicated in Table 6. The screening process selected 23 records accounting for fungi, host crops, environment and their interaction along the crop production chain. Regarding co- and occurrence in sorghum, 21 records passed the screening process, but only one has data from EU countries, while the remaining 20 have data from non EU countries. Due to the limited number of papers retrieved on co- and occurrence in sorghum in Europe, an additional literature search was performed. The literature search was extended to the timeframe from 2000 to 2009 and 2017-2018. Ten out of the nineteen additional records retrieved were selected after screening. However, of these ten only one record reported data from Europe. Thus, data on occurrence in sorghum were extracted from only 2 papers (Figure 6).

More details on the integrating ELS for sorghum are reported below. The following databases were used:

- MEDLINE®
- CABI: CAB Abstracts®
- PubMed®

The following terms were considered, to be present either in the title and abstract:

(((sorghum) AND (fung* OR fusarium OR aspergillus OR penicillium OR claviceps OR alternaria OR aaltb toxin OR alternariol)) AND (Mycotoxin* OR toxin* OR Aflatoxin* OR AF OR fumonisin OR FB OR deoxynivalenol OR DON OR ochratoxin OR OTA OR zearalenone OR ZEN OR ZEA OR patulin OR PAT OR T2 OR HT2 OR Trichothecene* OR ergot alkaloids OR beauvericin OR BEA OR Enniatin* OR ENN OR moniliformin OR MON)) AND (occur* OR co-occur* OR cooccur*)

The ELS library for **nuts** included 1560 records. The screening process selected 39 records accounting for fungi, host crops, environment and their interaction along the crop production chain and 3 regarding modelling. Regarding occurrence in nuts, papers passing the full text screening were 13 (5 with occurrence data from EU countries and 8 with occurrence data from non EU countries). Only the available papers with occurrence data from EU countries were extracted (Figure 5).

The ELS library for **grapes** included initially 1973 records, organized as indicated in Table 6. Following the same selection process, the number of selected records (after full-text retrieval and screening for occurrence data) was reduced to 239. Based on the information retrieved so far, it is possible to observe that most of the published works relates with the occurrence and the ecology of *Aspergillus* species (predominantly black *Aspergilli* species), the occurrence of ochratoxin A (OTA) in wines, and the inhibition of OTA production. Regarding mycotoxin occurrence, 33 records were retrieved, being 14 from countries outside the EU and the remaining 19 included in database (Figure 4).

In ELS library for **spices**, 553 records were initially present. The screening flow led to 20 records retrieved from the cropping search-group and only 1 related to modelling. Of the final 39 records with occurrence data, 30 were from countries outside the EU, remaining 9 to be included in database. Of these, studies included a wide range of spices, with different varieties of pepper being the most usually analyzed.

Records on **biosynthesis** were initially divided by crop. In order to have them by mycotoxin, they were merged in one Endnote TM 8 library. The final number of records after duplicates removal and screening (title and abstract) was 306. However, most of the papers deal with the biosyntehsis of single compounds. Therefore, the authors integrated the ELS according to their expertise, and focus on the formation of modified mycotoxins in plants. The authors also noticed that the factors regulating the interaction between fungi and the subsequent accumulation of multiple mycotoxins are still unknown so far.

Regarding **modelling**, 242 records were retrieved among the seven considered crop groups and, 53(21%) records passed the selection screening (full-text retrieval). 42 (80%) of the included records, concern maize and small grains, and 11 records (20%) regard the remaining crops (grapes (4), nuts (3), rice (3) and spices (1)). Among the small grain group, wheat is the predominant cereal crop with 18 records out of 25 (72%); 2 papers were found on oat. No record has been found in sorghum. The majority of retrieved records regard modelling fungi growth and mycotoxin production under *in vitro* condition, whereas our interest refers to modelling mycotoxin contamination in crops under field condition. Based on the information retrieved, it is possible to notice that no major improvement have been achieved on this topic since the last review by (Battilani et al., 2016). We confirm the focus in modelling mycotoxin in few crops, including maize and other cereals, mainly wheat. Minor efforts were devoted to aflatoxin contamination in pistachios and OTA in grapes.

2.3.2. WP2

This section presents the inclusion/exclusion criteria and the outcome of the ELS for the toxicokinetics, toxicity and biomarker studies.

Papers pertaining to WP2 were initially screened starting from toxicokinetics (TK), to set up common inclusion/exclusion criteria to be used for further refinement of the ELSs.

2.3.2.1. Toxicokinetic data

The screening of the papers providing toxicokinetic studies was based on the relevance of the papers with regards to absorption, distribution, metabolism, excretion, bioavailability and carry-over of selected mycotoxins in target species (as specified in the Section 2.2.2). The process consisted of two steps:

1. Screening of the titles and abstracts of all publications. Publications included at this stage, were analysed in the second step;
2. Screening of the full text of the publications included. Publications considered relevant, were examined for inclusion within the report.

In order to perform the screening in a harmonised way appropriated inclusion and exclusion criteria were adopted, as reported below

Inclusion criteria:

- The paper addressing toxicokinetic data for single and/or co-occurring mycotoxins
- Study design:
  -in vivo experimental laboratory studies
  -in vivo field/semi-field studies
  -in vitro model-based studies
- Outcome of interest:
  Data and information relating to the toxicokinetic effects of single or mixtures of mycotoxins (ADME, bioavailability, carry over)

Exclusion criteria

- Duplicated studies: the most recent one was usually carried to data extraction
- Studies reporting data on mixtures having a potential antagonistic interaction effect on toxicity (i.e. mycotoxin + quercetin). The outcome of the ELS for both *in vitro* and *in vivo* toxicokinetics is shown in Figure 8.
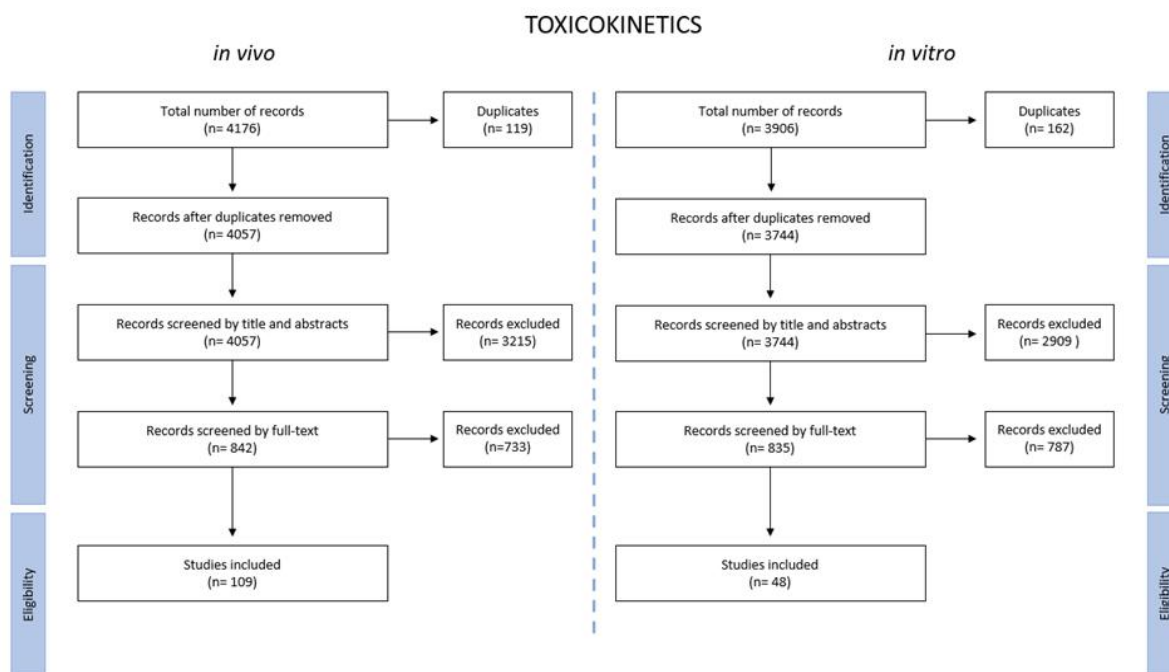
TOXICOKINETICS

Figure 8: Flow diagram on the study selection for toxicokinetics (*in vivo* and *in vitro*)

As complementary activity, UNIPR set up a template for data collection, to be used for literature meta-analysis and modelling. A spreadsheet format has been agreed for practical reasons (i.e. wider distributability and usability among the WP operators than other options). The template to collect toxicokinetic data *in vitro* and *in vivo* **(Appendix B.5 and B.6)** attached to this report is based on modifying the toxicokinetic OECD template to facilitate data mining and data extraction for modelling. It is highly detailed and allows for the collection of a large number of parameters.

In the final filtering, which covered also the full text of articles, the following strategy was applied:

Mixture data

- Collection of data from papers mentioning co-occurrence/co-exposure of mycotoxins
- Separation of papers by experimental animal species
- Identifying the papers with dose response data

Single compound data

- Division of papers by mycotoxin
- Separation of papers by experimental animal species
- Identification of papers with dose response data

2.3.2.2. Toxicity data

Concerning the filtering process for toxicity data, 7599 papers were identified in the ELS and were filtered on the basis of the most common occurring mycotoxins (deoxynivalenol, aflatoxins, fumonisins, zearalenone) as single compounds and their possible co-occurrence. Target species included the most relevant farm animal species: chickens, pigs and cows.

The inclusion criteria for the data extraction and to design the template for the data collection were defined as:

- in field, semi-field or laboratory *in vivo* study
- relevant endpoints for risk assessment
- toxicity assessed for single compound AND mixture
- multiple doses for a possible dose response (BMDL) assessment

Exclusion criteria were the same used for TK (i.e., duplicated studies and studies reporting mixtures with compounds having potential protective effects were not considered for the data extraction). In addition, *in vitro experiments* and studies addressing individual toxins assessment or mixtures assessment lacking the assessment of single compound effects were excluded too. The outcome of the papers screening regarding toxicity is shown in Figure 9 below.
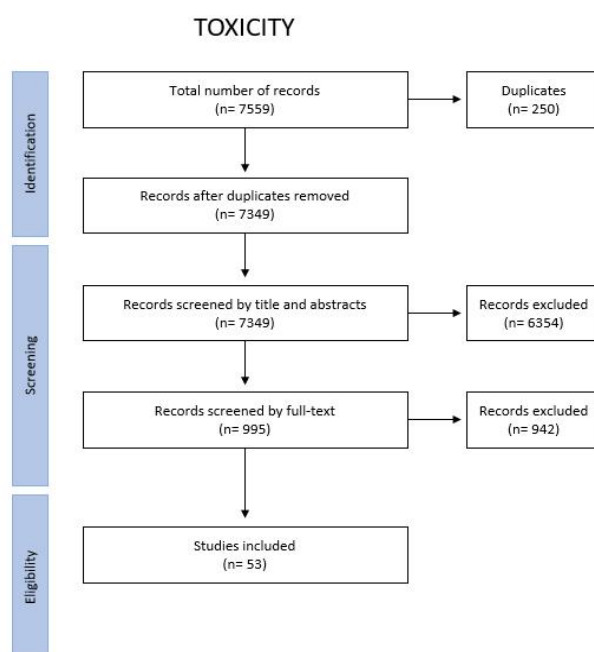
TOXICITY

Figure 9: Flow diagram on the study selection for *in vivo* toxicity

From the total number of papers only 53 papers matched the above-mentioned inclusion criteria although the papers identified included only single mycotoxin exposure. Therefore, an additional literature search before 2011 was done resulting in an additional set of relevant papers (n=65), including both single and mixture treatments and published between 1976 and 2010, which were not included in the ELS output. This selection of papers was used for the data extraction. As done for TK analysis, a template for data collection and modelling has been provided as a framework method tool. The templates developed to collect data are reported in **(Appendix B.1)**.

2.3.2.3. Biomarkers of exposure and effects

In this research, the extensive literature search retrieved a total of 5753 records that were submitted to the inclusion/exclusion criteria step. Figure 10 shows the outcome of the papers screening regarding biomarkers.

Following the exclusion/inclusion criteria, the following records were excluded:

- All studies on food analysis (ex. occurrence);
- All studies on toxicology (without biomarker info);
- All studies on biomarker of effect where the effects affected cell mechanisms or cellular metabolites or changes in protein pathway or DNA repair pathway and genetic expression;
- All studies on parameter associated to mycotoxins but not H/A exposure specific (fungal activity, plant activity);
- All studies on analytical methods not intended for biomarkers
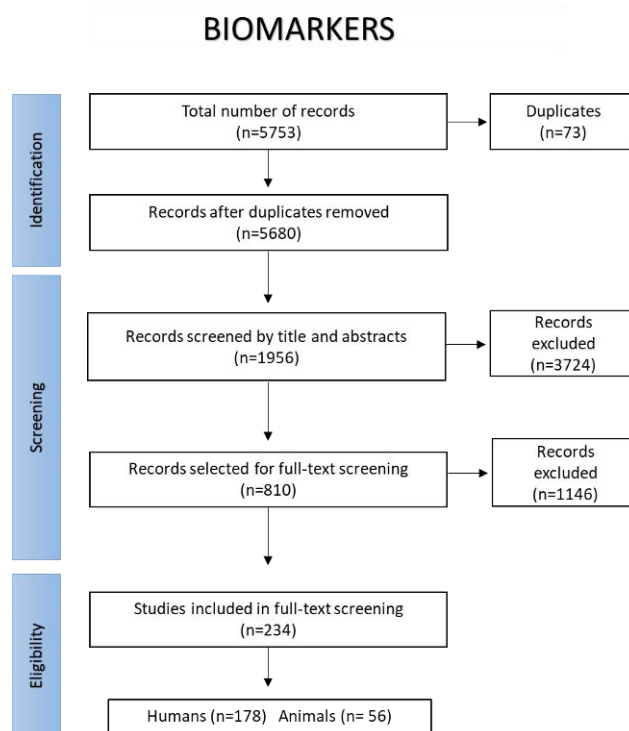
BIOMARKERS

Figure 10: Flow diagram on the study selection for biomarkers

The 86% (n=4943 over 5753) of the records were excluded as not inherent with the specific biomarker topic; while the rest of the records (n=810) where categorized as follows:

- Records reporting biomarkers of exposure information (human and animal) (70%)
- Records focused on studies about biomarkers of effect (41%)
- Records focused on toxicological study but containing biomarkers information (18%)
- Records focused on toxicokinetic study but containing biomarkers information (6%)
- Validation of the analytical method for biomarker analysis (5%).

Table 7: Percentages of papers found for each screening category divided for human and animal species (screening not completed)

Screening category	Human	Mice	Broilers	Pig	Dog	Cow beef
Biomarkers of exposure	58%	0	1%	45%	5%	2%
Biomarkers of effect	1%	0	0	0	0	0

Toxicological studies with data on biomarkers	10%	0	0	3%	4%	0
Toxicokinetic study with data on biomarkers	1%	2%	3%	0	0	0
Validation of the analytical method for biomarker analysis	4%	0	0	0	0	1%

Thus, all the included records were scrutinized to derive the list of indicators and parameters to be used. The list was shared with partners for further discussion and a final definitive list was established.

For the prioritisation of the biomarkers paper scrutiny, the following strategy was performed for both mixture and single biomarker data.

- Collection of data from papers mentioning biomarker measurements;
- Collection of all critical information from the paper;
- Separation of papers by human and experimental animal species

2.3.2.4. Analytical methods

A total of 12057 records retrieved from the literature were submitted to the inclusion/exclusion criteria step. The 55% (n= 6714) of the records were excluded as not inherent with the specific topic; while the included records (n=5343) where grouped in 11 categories, as shown in Figure 11.

Figure 11: Flow diagram of the study selection process for methods of analysis
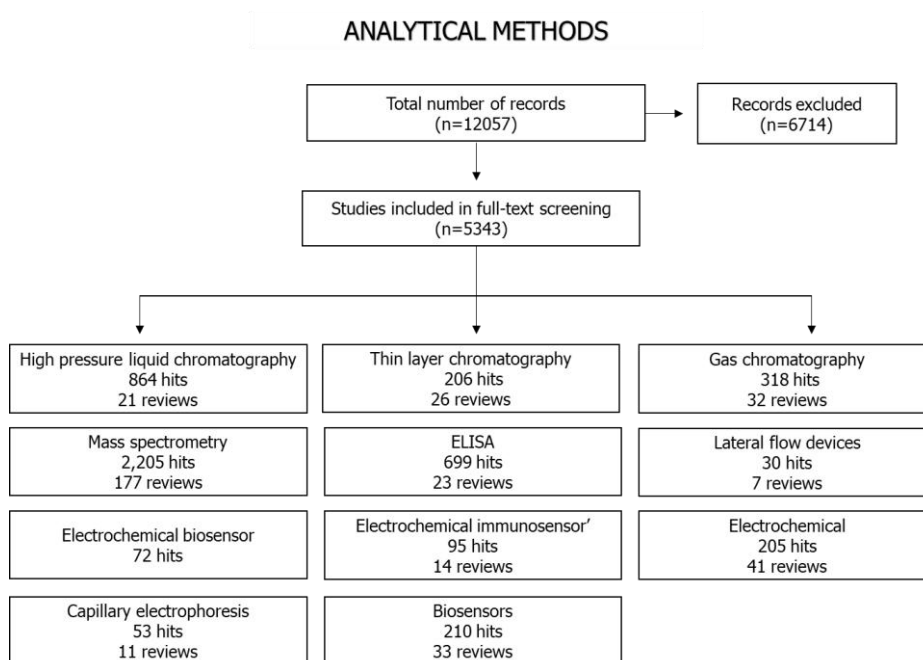
2.4. Data extraction

2.4.1. Data extraction on occurrence and co-occurrence

Data on occurrence and co-occurrence of mycotoxins in maize, small grains, rice, sorghum, nuts, grapes and spices were extracted from the included papers. Data extraction was performed partially manually or by using the tool called Tabula 1.1.1. A data model for data collection on mycotoxins occurrence/co-occurrence at aggregated level was developed according to EFSA requirements **(Appendix A.1)**. The aim of this collection was to review available information on occurrence/co-occurrence to be used for further analysis. Moreover, UCSC prepared a second excel table for data collection at sample level according to the SSD2 standards, to be performed at a later stage and submitted to EFSA for validation. However, the restricted number of studies retrieved on occurrence data at sample level, did not allow any further analysis.

2.4.2. Data extraction on toxicity and toxicokinetics

For the prioritisation of the toxicity and toxicokinetic studies the following strategy was performed.

- Mixture data
    - Collection of data from papers mentioning co-occurrence/co-exposure of mycotoxins
    - Separation of papers by experimental animal species
    - Identifying the papers with dose response data

- Single compound data
    - Division of papers by mycotoxin
    - Separation of papers by experimental animal species
    - Identification of papers with dose-response data

To represent the type and amount of data available and in accordance with the aforementioned criteria, two tables were prepared **(Appendix B.3 and B.4)**.

Template forms for data extraction, structured according to the relevance of the data provided and based on the outcomes of interest were developed. The extraction grids were prepared separately for each literature search and for each relevant endpoint (toxicity, toxicokinetics *in vitro* and toxicokinetic *in vivo*).

2.4.2.1. Toxicity

After a preliminary screening of the selection of papers, the template was outlined taking into consideration the relevant toxicological endpoints accounted in the studies. The original design aimed to provide an overview of the studied endpoints, summarizing each study in a single row. A posterior version of the template allocated each dose group to an individual row for higher detail. In the final version of the template numerical values and unit of measurement were included in the design to facilitate data mining and modelling. As mentioned in section 2.3.2.2 an additional search was performed for single and mycotoxin mixture studies covering the years before 2010, both templates are presented below and examples can be found in the **Appendix B.1 and B.2**.

The template for the toxicity studies ranging between 1976 and 2010 included the following:

- The first columns present the toxin being tested as well as the study in question: "Toxin", "DOI" and "Author & year";

- The following columns give information on the species, exposure time and route of administration: "Production (Purity)", "Origin", "Species", "Duration of exposure", "Exposure" and "Route of administration", "Dose", "Replicates", "Number of animals" and "Sex";
- The next columns reflect on toxicological endpoints such as "Weight", "Microscopic findings", "Macroscopic findings", "Haematology", "Biochemistry", "Cancer incidence" and "Mortality rate". Parameters typically included in toxicity which will be detailed in the next criteria.

An example of the template columns including information extracted from the literature can be found in the **Appendix B.1**.

The template for toxicity studies ranging between 2010 and 2017 included the following:

- The first columns include details on the study ("Author", "Year", "Title")
- Columns covering species information ("Species", "Strain", "Sex", "Initial age", "Initial body weight", "Sample size")
- Mycotoxin information including dose and exposure ("Mycotoxin", Time of exposure", "Dose in the feed", "Dose", "Exposure feed/gavage")

Toxicological endpoints including the observed effect values ("Effect", "NOAEL", "LOAEL", "Mean observed value", "Statistical descriptor".

2.4.2.2. Toxicokinetics

The purpose of the template for toxicokinetics *in vivo* was the collection of all the relevant quantitative and qualitative TK parameters in order to feed the predictive models.

- The first columns present the reference, the experimental animal with its "sample size", the "administrated dose" (in harmonised units), the "toxin source" and its "route of administration".
- The following columns represent relevant TK parameters relevant to absorption like the "per cent of the total absorption" and "bioavailability (%)".
- Additional grids are added for the metabolic and distribution criteria; "t1/2 el": the time it takes for the concentration of the compound to decrease by 50%, "AUC": area under the plasma (blood) level vs. time curve from zero up to a certain measured time point, "Cmax": maximum (peak) concentration "Tmax": time to reach peak or maximum concentration following administration, "Vmax": maximum elimination capacity, "Km": concentration at 50% of Vmax
- The next columns address excretion parameters like; "excretion route", "% of dose excreted", "excretion time observed".
- Finally, the last grids of the template refer to "carry-over rate", "target tissue" "toxin concentration" and the "analytes considered for evaluation".

Analogously, the purpose of the template for toxicokinetics *in vitro* was the collection of relevant aspects of mycotoxins kinetics such as:
- Cell/tissue type, or cell organells involved in the process
- Types of transports, exposure time and excretion rate
- Production of phase I and phase II metabolites, and respective transformation yield
- Transforming enzymes (e.g. CYPs)
- Kinetic parameters such as K_i, K_m, permeability, intrinsic clearance (Cl_{int}), maximum bioavailability (f_{max}) and V_{max}

2.4.3. Data extraction on biomarkers

After sharing and adjusting for the most appropriate format with the kind of indicators of interest, the following info/group of information were identified to be registered from each record to feed the dataset:

- Reference
- Information about the biomarker and the study (Type of study, Mycotoxin, Biomarker, Substrate, Country, SampleSize, SampleType, Age, SamplePeriod)
- Information on the quantification and statistics of the biomarker (Mean, Median, GeometricMean, SD, Percentile, Percentile_ INFO, Unit, Min, Max, Creatinine/Albumine corrected, Biomarker Creatinine/Albumine, Biomarker creatinineUnit, Biomarker_INFO)
- Information about the analytical methods (Analytical method, Method Validation, Method Validation_INFO, Method LOQ, Method LOD, LOQunit, LODunit, Other_parameter_ labeledstandards_INFO_NOTA
- Type of sampling strategy used
- Information about the correlation with food intake or exposure assessment calculations (Correlation_food, Any correlation with food intake, Exposure assessment, ExpAss_Value, Expass_Unit

- Information on biomarker validation process
- Other notes

The comprehensive indicator list is reported in **(Appendix B.5)**. The list of recurrent indicators and explanation retrieved is reported in **(Appendix B.6)**.

The template characteristics were defined to record one line for each biomarker and to ensure:

- an easy legible content with the best information
- relevant information of the selected indicators
- a broad scope; kind of additional information under INFO columns was added to report useful details.

2.5.   Database development

2.5.1.   Development of a structured database across the WP1-2

All data extracted for mycotoxin occurrence/co-occurrence (**Section 2.4**) were stored in proper databases and interfaced to R environment for statistical computing and graphics. **Appendix A.1** provide a comprehensive data elements description about the database as a whole for occurrence and co-occurrence of mycotoxins in maize, small grains, rice, sorghum, grapes, nuts and spices.

This approach has been shared with all project partners and the following description is also common for the in-progress databases related to toxicity, toxicokinetics *in vitro,* toxicokinetic *in vivo* and biomarkers (**Appendix B.1, B.2, B.3 and B.6**). This common approach enables the data analysis through a set of R functions to be able to find, access and use data and it will facilitate modelling implementation (WP3). All the DB files and functions are currently available on the MYCHIF project repository and EFSA knowledge junction (10.5281/zenodo.3615174).

Appendix C.1 and **C.2** provide, respectively, the list of abbreviations and encoding keys.

2.5.2.   Data Analysis

The following query keys were assigned to extract data and perform a qualitative and a first quantitative analysis assessment:

each "PARAMTYPE" & each "sampMatbased" & each "sampMatType"

where "PARAMTYPE" are all the toxins reported in **(Appendix C.2)**.

where "sampMatbased" are maize, rice, grapes, almond, barley, buckwheat, caraway, cereals, durum wheat, millet, oat, peanuts, rye, soft wheat, soy, spelt, triticale, walnuts, wheat, glutenfree

where "sampMatType" are food, feed and raw materials

For each data extraction a ECDF object and plot (empirical cumulative distribution function) has been created (Figure 12) for the "Concentration" value and if not available for the "meanTot" value. All the ECDFs plots are available on MYCHIF project repository and EFSA knowledge junction (10.5281/zenodo.3615174). Two kinds of ECDFs are available: a discrete ECDF plot (single points, Figure 13) and a fitting ECDF plot (Figure 12).
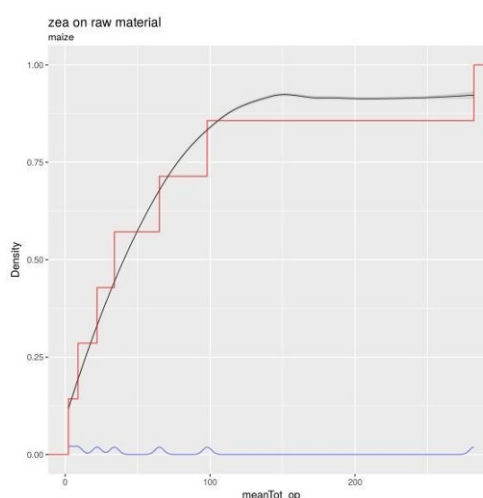
Figure 12: fitting ECDF example for Maize+ZEN+Raw material, "meanTot" value

zea on raw material
maize
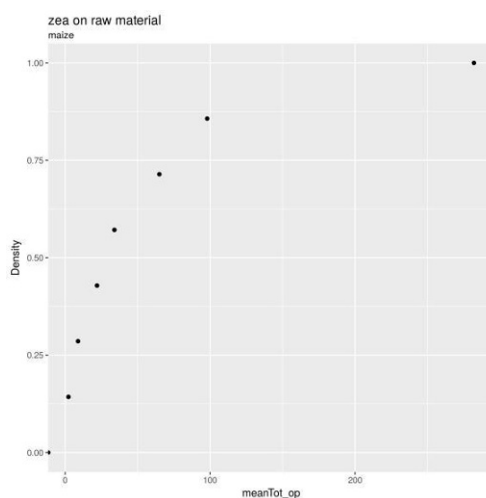
Figure 13: simple ECDF example for Maize+ZEN+Raw material, "meanTot" value

After the ECDF analytics, the first basic step in a Quantitative Risk Assessement (QRA) is to generate amplified data by using a Monte-Carlo simulation (R package mc2d) that considers the parameter (toxin) as a random variable having a predefined theoretical probability distribution. Considering that each parameter is both uncertain and variable, we have specified a bounded set of candidate parametric distribution (Poisson, lognormal, exponential etc.) and obtained distribution plot representing the potential variability of parameters investigated (Figure 14 and Figure 15).

QQplot MC DON on raw material - Maize
Nvar=1000 Nunc=100 N=6
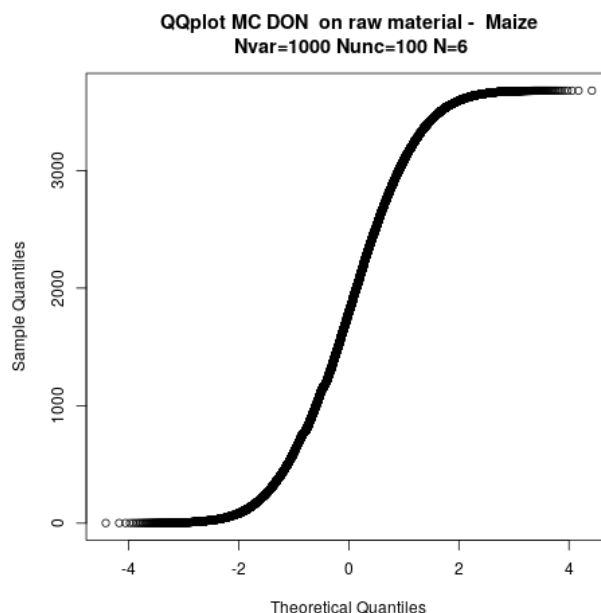
Figure 14: QQplotfor DON+RawMaterial+Maize, "concentration value", a graph of the cumulative empirical distribution function of the estimate of the quantiles
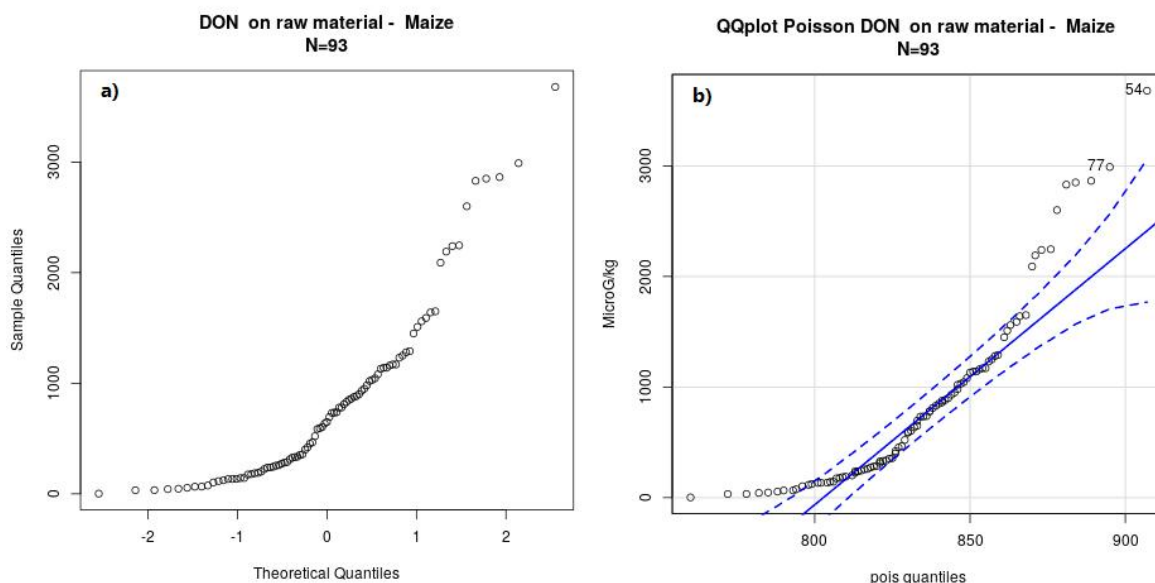
Figure 15: QQplot for DON+RawMaterial+Maize, "concentration value", a) theoretical quantiles; b) Poisson parametric distribution

Furthermore, for each data extraction a summary table has been exported with the following data (Table 8).

Table 8: An example of data extraction summary, maize

Keys	Concentration Mean	ToTresVal UncertSD_Mean	meanTot	POSresVal UncertSD	Mean Pos	min	max	LOD	Tot sample	Record	Nsample concentration	Nsample mean Tot
afb1; raw material; maize		36.10	8.70			6.20	33.27	22.83	242	6	0	6
afb1; feed; maize		47.35	9.80				345.31	44.00	197	5	0	4

(a): Keys: the assigned keys for data extraction; Concentration_Mean: mean of total samples; TotresValUncertSD_Mean: mean of standard deviation of total samples; meanTot: mean of mean total samples; POSresValUncertSD: mean of standard deviation of positive samples; meanPos: mean of mean positive samples; min: minimum of minimum total samples; mas: maximum of maximum total samples; LOD: mean of total LOD samples (%); tot sample: number of total samples; Record: number of total records; Nsample_concentration: number of samples with concentration value; Nsample_meanTot: number of samples with meanTot value.

Regarding co-occurrence analysis, a first attempt of analysis has been performed, data extraction rules are based on the following keys:

each "sampMatbased" & "sampMatType" & "Co-occurrence=1" & each "Ref" & each "SampSize"

where "sampMatbased" and "sampMatType" are the same as defined above, "Co-occurrence=1" means only samples where co-occurrence has been reported, "Ref" and "SampSize" are the papers and the size of samples to group only the same samples data.

All the tables are available on MYCHIF project repository and EFSA knowledge junction (10.5281/zenodo.3615174).

Table 9: An example of co-occurrence table analysis, grapes

samp Matba sed	samp Siz e	paramType	samp Country	sampCo untryori gin	Reco rds	Ref	samp MatTy pe
grapes	1	AOH+AME+ALT+ATX1+ATX2+ TEA+ macrosporina+TEN	SK	SK	104	(Mikusova, Sulyok, & Srobarova, 2014)	food
grapes	1	emodin+malforminc+3np+kojic acid	SK	SK	48	(Mikusova, Sulyok, Santini, et al., 2014)	food
grapes	12	festuclavine+OTA+FB2	SK	SK	3	(Mikusova, Sulyok, Santini, et al., 2014)	food
grapes	1	atx1+alterperylenol+atx2+AOH +AME+TEN	DE	DE	6	(Liu & Rychlik, 2015)	food
grapes	1	OTA+OTB+AFB1+AFG1+AFB2 +AFG2	IT	IT	180	(Stefano et al., 2015)	food
grapes	1	FB2+FB4	DK;DE;NL	US;TR;G R;ZA;CN; CL	48	(Knudsen Peter et al., 2011)	food
grapes	4	OTA+FB2	GR	GR	4	(Perrone et al., 2013)	food
grapes	6	OTA+FB2	GR	GR	2	(Perrone et al., 2013)	food
grapes	18	OTA+FB2	GR	GR	2	(Perrone et al., 2013)	food
grapes	9	OTA+FB2	GR	GR	2	(Perrone et al., 2013)	food
grapes	1	AFB1+OTA+AFB2+AFG1+AFG2	PL	CL;IR;TR	25	(Jeszka-Skowron et al., 2017)	food
grapes	35	OTB+OTA+MEOTA+OTC+MEO TB+ETOTB	ES	ES	6	(Gonzalez-Penas et al., 2012)	food
grapes	16	OTB+OTA+MEOTA+OTC+MEO TB+ETOTB	ES	ES	6	(Gonzalez-Penas et al., 2012)	food
grapes	71	AFB1+AFB2+AFG1+AFG2+BEA +DAS+ENB+ENB1+ENA+ENA1 +HT2+OTA+T2+FB1+FB2+FB 3	TN;ES	TR;IR;CL ;DZ	16	(Azaiez et al., 2015)	food

R packages

The following R libraries and packages have been used for data import, visualization and analysis:

library(readxl)

library(XLConnect)

library(ggplot2)

library(dplyr)

library(scales)

3. Results

3.1. Mycotoxin producing fungi

Worldwide, the most relevant and studied mycotoxins are synthesised by fungal species belonging to three genera: *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp., and on a minor extent by *Alternaria* spp. and *Claviceps* spp. Mycotoxigenic fungi are commonly not host specific, but their occurrence is mainly signalled in a specific crop depending on its growing area and meteorological conditions (Table 10).

Table 10: Some fungal species of interest with corresponding mycotoxins and their primary crop hosts

Fungi source	Mycotoxins[a][b]	Crops of primary concern
***Aspergillus* spp.**		
A. carbonarius	OTA	grapes, pistachio nuts
A. flavus	AFB1, AFB2, STC	maize, rice, grapes, sorghum, nuts (mainly peanuts, pistachio and almond nuts)
A. niger	OTA, FB2	grapes, sorghum, nuts (mainly peanuts, pistachio and almond nuts)
A. ochraceus	OTA	rice, grapes, nuts
A. parasiticus	AFB1, AFB2, AFG1, AFG2, STC	maize, nuts (mainly peanuts, pistachio and almond nuts)
A. tubingensis	OTA	sorghum
A. versicolor	STC	rice
***Fusarium* spp.**		
F. armeniacum	T-2, HT-2	rice
F. avenaceum	ENNs, MON	small grains (mainly wheat, barley, oat), grapes
F. culmorum	DON, AcDONs, NIV, ZEN	maize, small grains (mainly wheat, barley, oat)
F. equiseti	ZEN	maize, small grains (mainly wheat, barley, oat)
F. fujikuroi	FB1, MON, GA	rice
F. graminearum	DON, AcDONs, NIV, ZEN	maize, small grains (mainly wheat, barley, oat), rice
F. incarnatum	ZEN	sorghum
F. langhsethiae	T-2, HT-2	maize, small grains (mainly wheat, barley, oat)
F. nivale	NIV	small grains (mainly wheat, barley, oat)
F. oxysporum	ENNs, MON, BEA	grapes
F. poae	NIV, T2- HT2, DON, AcDONs, DAS, ENNs	small grains (mainly wheat, barley, oat)
F. proliferatum	FBs, BEA, FUS, MON	maize, small grains (mainly wheat, barley, oat), rice, grapes, sorghum
F. pseudograminearum	DON	small grains (mainly wheat, barley, oat)
F. pseudonygamai	FBs	sorghum
F. sporotrichioides	T-2, HT-2	maize, small grains (mainly wheat, barley, oat), grapes
F. subglutinans	BEA, FUS, MON	maize, rice
F. temperatum	FBs, BEA, FUS, MON, ENNs	maize
F. thapsinum	ZEN	sorghum
F. verticillioides	FBs	maize, grapes, sorghum

Penicillium spp.		
P. aurantiogriseum	PAC	rice
P. citreonigrum	CTN	rice
P. citrinum	CIT	rice
P. commune	CPA	rice
P. expansum	PAT, CIT	grapes
P. islandicum	CC, uteoskyrin	rice
P. rugulosum	Rugulosin	rice
P. verrucosum	CIT, OTA	wheat, rice, grapes
Alternaria spp.		
A. alternata	ATs	grapes
A. infectoria	ATs	rice
A. tenuissima	ATs	rice
Claviceps spp.		
C. africana	EAs	sorghum
C. purpurea	EAs	small grains (mainly wheat, barley, oat)
C. sorghi	EAs	sorghum
C. sorghicola	EAs	sorghum

(a) AFs: aflatoxins; AFB1: aflatoxin B1; AFB2: aflatoxin B2; AFG1: aflatoxin G1; AFG2: aflatoxin G2; ATs: Alternaria toxins; BEA: beauvericin; CC: cyclochlorotin; CIT: Citrinin; CPA: cyclopiazonic acid; CTN: citreoviridin; DON: deoxynivalenol; AcDONs: acetyldeoxynivalenols; EAs: ergot alkaloids; ENNs: enniatins; FBs: fumonisins; FB1: fumonisin B1; FB2: fumonisin B2; FUS: fusaproliferin; DAS: diacetoxyscirpenol; GA: gibberellic acid; HT-2: HT-2 toxin; MON: moniliformin; NIV: nivalenol; OTA: ochratoxin A; PAC: penicillic acid; PAT: patulin; STC: sterigmatocystin; T-2: T-2 toxin; ZEN: zearalenone

(b) the complete list of mycotoxin abbreviations is provided in **Appendix C.2**

Temperature (T), relative humidity (RH), rainfall (R) and, above all, water activity (a_w) are the most important ecological factors influencing fungal colonisation of the substrate, and every species has its peculiar ecological needs. Fungal growth can occur in a wide range of a_w and T and, optimal conditions for fungal growth do not always correspond to those appropriate for mycotoxin biosynthesis. Furthermore, host crop-fungi interaction impacts on the ecophysiology of fungi; therefore, it is difficult to withdraw summary information. For a general overview, main findings regarding T and a_w range for fungal growth and mycotoxin production in the most relevant fungal species is illustrated in Table 11 and Table 12.

Table 11: Temperatures (°C) needed for the fungal growth and mycotoxin production for same of the most relevant species isolated in different crops
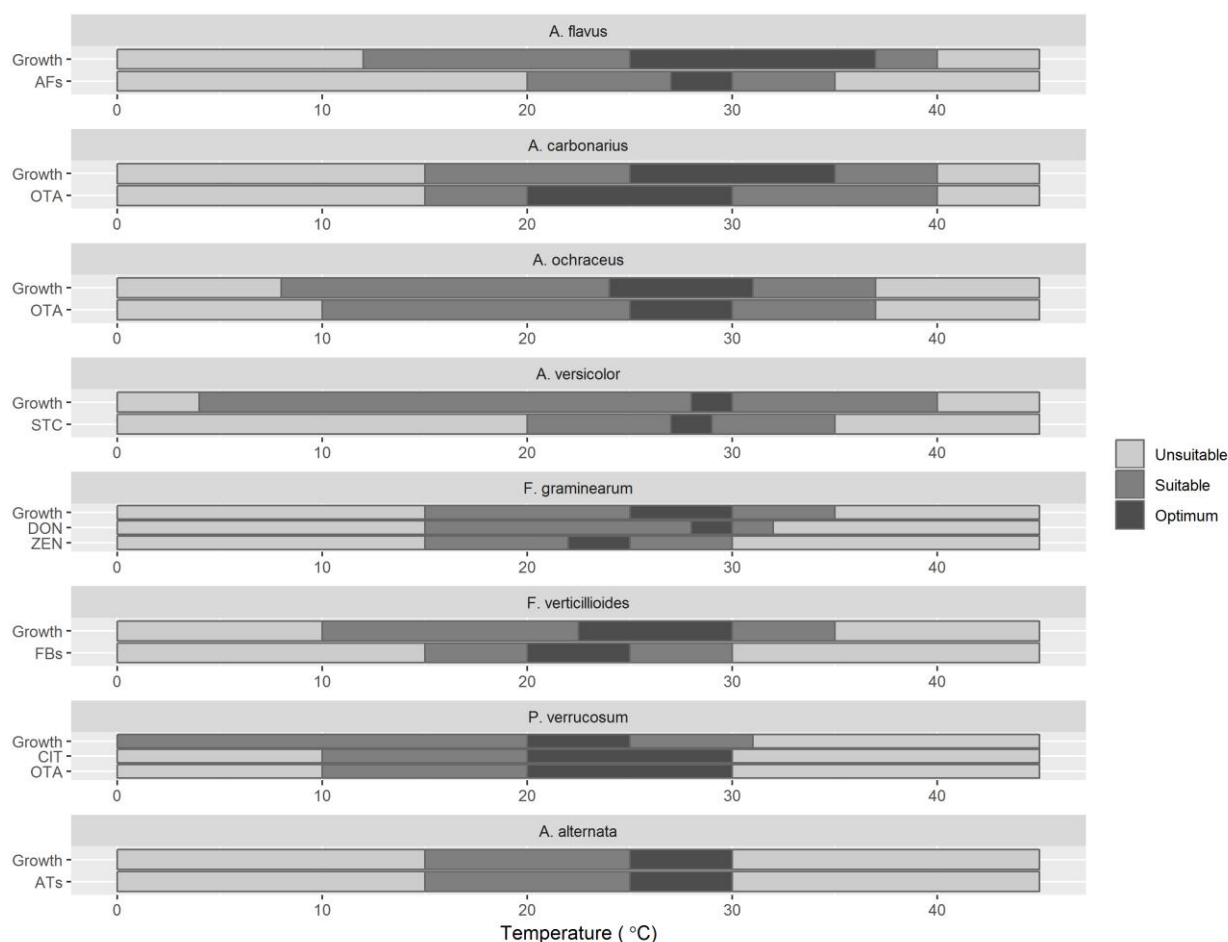
Data on ecological needs are available for several Aspergilli, *A. flavus, A. carbonarius, A. ochraceus and A. versicolor. A. versicolor* is reported as the most flexible in term of T requirements, being able to grow from 5 to 40 °C; *A. carbonarius* has the narrower useful T range, from 15 to 40°C. 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. *Fusarium graminearum* and *F. verticillioides* are the most important species among Fusaria. *F. verticillioides* is known to be more thermophilic compared to *F. graminearum*, but they have comparable suitable T ranges for growth. Optimum for FBs production is switched towards lower temperature respect to fungal growth. Interesting for *F. graminearum* is the difference between optimal conditions for DON and ZEN production, the former close to 30°C the latter below 25°C. Penicillia are less thermophilic compared to the other genera and *P. verrucosum* can grow from 5°C, with toxin production from 10°C, at least CIT.

Table 12: Values of the water activity (a_w) needed for the fungal growth and mycotoxin production for same of the most relevant species isolated in different crops
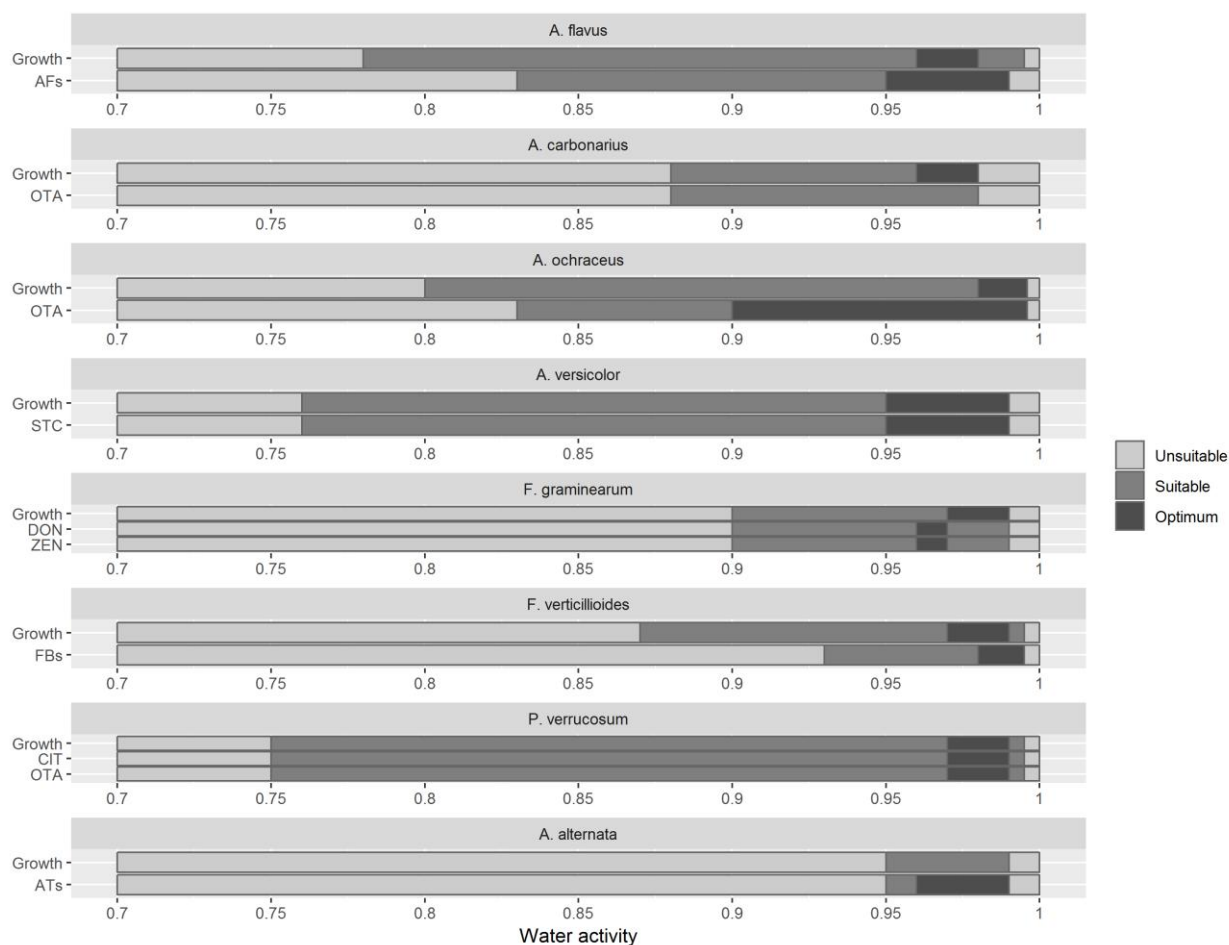
3.1.1. Good agricultural and management practices

Managing mycotoxin contamination requires a comprehensive strategy that includes a correct pre-harvest management and good harvest and post-harvest procedures. Figure 16 summarizes the effect of the main growing, handling and processing steps in the occurrence and fate of mycotoxins for the main crops included in this report ((Gonçalves et al., 2019; Gonçalves et al., 2020; Palumbo et al., 2020b); **Annex A-E**).

		Maize				Small grain				Rice					Nuts	Grapes			
		AF	FB	DON	ZEN	DON	ZEN	T2-HT2	Ergot	FB	AF	OTA	CIT	STC	AF	AF	FB	OTA	PAT
Pre-harvest	Sowing time	Y	Y	G	G	G	G	G	Y	—	—	—	—	—	Y	—	—	—	—
	Plant density	Y	—	Y	Y	Y	Y	G	—	—	—	—	—	—	Y	Y	Y	Y	—
	Genotype	Y	Y	Y	Y	G	Y	Y	—	—	Y	—	—	—	Y	Y	Y	Y	Y
	Soil management	G	G	—	—	G	G	G	R	—	Y	—	—	—	—	Y	Y	Y	Y
	Irrigation	R	R	G	Y	G	G	G	—	—	Y	—	—	—	Y	Y	Y	Y	Y
	Weeds control	Y	Y	G	G	—	—	—	—	—	Y	—	—	—	—	G	—	R	—
	Pest control	G	R	G	G					—	Y	—	—	—	R	Y	Y	Y	Y
	Fungal control (chemical)	Y	G	G	—					—	Y	—	—	—	G	Y	Y	Y	Y
	Fungal control (biocontrol)	Y	—	—	—	Y				R	Y				R	Y	Y	Y	Y
Post-harvest	Drying	R	—	—	—	R	R	R	G	R	R	R	G	R	R	R	R	R	R
	Cleaning and sorting	R	R	R	R	R	R	Y	R	R	R	R	G	Y	R	G	G	G	G
	Storage	R	R	R	R	R	R	Y	—	R	R	R	G	Y	R	R	R	R	R
	Processing	R	Y	Y	—	R	Y	R	—	Y	Y	Y	1	Y	—	Y	Y	Y	2
	Detoxification	Y	Y	—	—	Y	Y	—	—	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Legend
- **Red** — effect on mycotoxin content
- **Yellow** — intermediate effect on mycotoxin content
- **Green** — no effect on mycotoxin content
- **Grey** — no studies/not (yet) applicable

1- only for Red Yeast Rice
2- removal of the mycotoxin in fermented products

Figure 16: Table reporting the effect of good agricultural and management practices pre- and post-harvest and their impact on the occurrence and fate of mycotoxins in the food and feed chain

3.1.1.1. Pre-harvest management

Several research efforts have defined good agricultural practices (GAPs) to apply during pre-harvest, including: (i) choice of variety/hybrid, (ii) soil management, (iii) burial of crop residues and crop rotation, (iv) control of plant stresses, (v) control of toxigenic fungi and (vi) restrain of pest insects (Battilani et al., 2012).

Sowing time

Fungi infection and mycotoxin contamination depend on the fungal inoculum, the co-occurrence of a susceptible plant growth stage, favourable environmental conditions, and vector activity. These factors are correlated with the sowing period, and therefore setting the right planting date can significantly influence the mycotoxin risk.

In maize, an earlier sowing date generally results in a lower risk of FB and AF contamination across diverse locations (Blandino et al., 2009). It is recommended to plant when there are good agronomic and meteorological conditions (soil temperature at least 10 °C since few days and sow 5 cm in depth); they allow an optimal root development making the plant less susceptible to water stress.

Plant density

Planting density affect mycotoxin risk in two possible ways: i) high density (more than 20% compared to the reference density for the crop/hybrid) affect water consumption causing water stress in plants, increasing their susceptibility; and ii) create a favourable microclimate for fungi development. It is therefore recommended to avoid forcing plant density to prevent water stress, especially in soils with low fertility and poor water availability.

Genotype (Variety – Hybrid)

The first stage of plant active defence is the detection of invading microorganisms, which is operated by plant receptors located both in the cytoplasm and on the plasma membrane. It is generally considered that comprehensive knowledge of plant defence mechanisms may help to identify resistant mechanisms, and assist the development of targeted and innovative approaches for breeding crop resistance. Thus, plant breeding has been used during the past years as a tool to develop maize lines resistance to abiotic and biotic stresses. However, no hybrids were found to be completely resistant to either fungi infection and/or mycotoxin

contamination because of the need to select for multiple traits and associated genes that contribute collectively to plant resistance.

In maize, consistent efforts were devoted and several research outputs are available regarding plant-pathogen interaction and host resistance; these are very promising starting points for future developments, but clear suggestions regarding hybrid selection, considered the best prevention tool, is not actually feasible.

More successful stories can be told regarding wheat, where resistant, or at least moderately resistant cultivars are available.

Soil management

Soil conditions influence plant root development. Crops with a poorly developed root system are more susceptible to stress, especially in sandy soils. Therefore, it is suggested to take care of soil drainage, especially in clay soils. It is also suggested to adapt tillage strategies to soil conditions to avoid drought stress. Tilled fields tend to dry more quickly and, under some circumstances, this drying can increase drought stress. Under other conditions, tillage may instead improve water availability to the plants by disrupting the compacted layers in the soil profile. Furthermore, cold soils can slow crop development. The resulting delay of flowering promotes the development of *Fusarium verticillioides* (FBs), while delayed ripening favouring *Fusarium graminearum* (ZEN and DON).

Mycotoxin producing fungi survive in soil or in crop residue on soil. Crop residue is the most important source of inoculum for *A. flavus* and several *Fusarium* spp. Crop rotation and tillage are good agricultural practices, recommended to reduce the inoculum of fungi overwintering in crop residues. In general, the efficacy of crop residue management varies by pathogen, by location and by the combination with other strategies. In small cereals, soil tillage and crop rotation managements are considered to be of major importance on mycotoxins control because of the splash borne dispersal of *F. graminearum*. On the contrary, *A. flavus* spores are air-dispersed, which means a long-distance distribution, and *F. verticillioides* is both air and splash dispersed. In this case, crop residue and soil management have almost no impact on fungal inoculum because it can arrive from neighbour fields.

Aspergillus carbonarius mainly survive in soil, with a minor contribute of grape crop residue and it is air dispersed. Therefore, similarly to other cited fungi, it is not significantly influenced by tillage in case other vineyards are around as possible inoculum source.

Irrigation

Even when irrigation as a mean of avoiding drought and consequent yield losses is required, its impact on *Aspergillus* and *Fusarium* development needs to be taken into account, as irrigation leads to an increased moisture, that can favour fungal growth, but it is well documented that limited water availability predisposes plants to AF contamination (especially relevant in maize).

In maize, with regards to *A. flavus* infection, water stress is particularly critical during silk emergence and kernels ripening. For FBs there is not such a clear evidence.

In wheat, irrigation should be minimized specially during anthesis, which is the most susceptible phase for Fusarium head blight (FHB) fungal infection. This was confirmed in wheat experiments with less FHB development observed when less irrigation was applied; however, late-season irrigation may reduce toxins possible due to leaching of these substances (**Annex B**; (Gautam and Dill-Macky, 2012)).

Weeds control

Weeds compete with the crop for water and nutrients, increasing the probability and magnitude of stresses. Therefore, it is suggested to apply accurate and timely weed control in pre or post-emergence, preferably integrated with the mechanical control on the row. Weeds may also act as a vector for cross contamination between crops (main vector for *Claviceps* spp. infection).

Pest control

Insect damages are well known to cause significant losses in yields and to be conducive of toxigenic *A.* section *Flavi* and *Fusarium* infection. Insects in the Order *Lepidoptera* typically have the biggest impact on mycotoxin-producing fungi. In particular, there are strong evidences of the big role played by the European corn borer (ECB), *Ostrinia nubilalis*, in *Fusarium verticillioides* infection. In small grains this action is mainly due to aphids and, particularly in wheat, *F. graminearum* spread can be double when plants host these insects.

In grapes, *Lobesia botrana* was shown to increase consistently OTA contamination; therefore, pest control is a very effective preventive action.

Fungal control (chemical)

Reported results are not conclusive regarding the use of fungicides to mitigate mycotoxin contamination in cereals. Several positive data have been published on the *in vitro* effect of fungicides on several pairs fungi-crop, but these results were not always supported by field trials.

In fact, the use of fungicides was ineffective in controlling *A. flavus* infection of maize at it showed a very limited effect against *F. verticillioides*. An important consideration, when deciding to use fungicides, is the possible effect on fungal population. Since the use of fungicides can promote the reduction of antagonistic fungi and contribute to more conductive conditions for pathogenic strains.

The most successful experience regards triazole fungicide treatments to lower DON contamination in wheat at the desirable level; however, in susceptible cultivars most fungicides fail to reduce mycotoxins levels to a desirable value.

The application of natural compounds is achieving increasing recognition as a potentially eco-friendly alternative to the use of synthetic chemical pesticides instead.

Fungal control (biocontrol)

The optimization of agricultural management practices to reduce the effects of drought and heat stress can reduce the gravity of aflatoxin contamination in maize, but these techniques are commonly insufficient. During the last decade, the biological control of pathogenic *Aspergillus flavus* has rapidly garnered the scientist's attention becoming the only recognised effective method for aflatoxin mitigation in some areas of the USA, Africa and Italy. This technique is based on the use of nontoxigenic isolates of *A. flavus* which act through competitive exclusion of aflatoxin producers in the environment and during crop tissue infection. It is applied for prevention of AF in maize, peanuts and pistachio nuts.

Far less information is available for the control of *Fusarium* spp., and for other crops, but promising results were produced regarding the prevention of Fusaria infection through the distribution of biological control agents on crop debris in small grains.

3.1.1.2. Harvest management and in-farm initial handling of crops

Fungal infection and subsequent production of mycotoxins begins in the field during plant growth and may continue through harvesting, storage, and processing if ecological conditions stay suitable for fungal activity. Mycotoxins, as stated before, are very stable compound and cumulate in time, both during crop growth and post-harvest. Therefore, the adopted procedures must always have this concept in mind. Factors as harvest time, sanitation conditions of the crop, and harvest techniques play an important role in the fate of the crop throughout its life time.

In cereals, grain moisture, or a_w, is crucial for fungal activity and it is directly influenced by harvest timing. Thus, it is good practice to harvest at moisture levels low enough to prevent post-harvest production of mycotoxins. This is commonly applied for wheat and small grain, with Fusaria as mycotoxin producing fungi of interest, because their activity is stopped by a_w decrease. In maize, *A. flavus* is of crucial interest and its activity continues till to 0.78 a_w, becoming very efficient in AF production when grain humidity is below 28%.

Therefore, it is suggested to balance between the risk of strong increase in contamination and harvest with suitable humidity for storage in defining harvest time and promptly artificially dry.

It is also important to test the field, or better predict with modelling approaches, for fungi contamination before harvesting, thus field with high risk of mycotoxin contamination should be harvested sooner, although with a_w higher than that suitable for storage, and dried immediately. Techniques applied from cutting during harvest may cause increased levels of fungal contents, with all the undesirable consequences. An effective crop management should allow harvesting at the right moment to assure the intended grain development and the reduction of contamination risks. Since a later harvest period is associated to a higher pathogenically fungal load, recorded for barley (Chen et al., 2016), and should be avoided.

Regarding fresh fruits, like grapes, harvest time is based on criteria related to the crop production quality, more than on mycotoxin contamination risk, but the stability and additive accumulation of these toxic compounds cannot be forgotten. This is of particular relevance for dried vine fruits (Gonçalves et al., 2020), and may be also considered for similar products (*e.g.*, dried figs).

Both for dry and fresh product, harvest should be managed avoiding as much as possible, damages to products, grain or fruit. In fact, mycotoxin producing fungi are commonly weak parasites and they take advantage of openings to easily enter and rapidly activate their metabolism.

A further point which should not be overlooked is the beneficial impact of operating a properly sanitation of load compartment used for the transport of harvested crop. In fact, dirty trucks with residue of contaminated crop may also be the source of risk contamination of the new load.

Depending on the crop and on-site facilities, initial handling of crops may include a few processing steps (as drying or sorting), before storage and commercialization.

Drying

Being moisture content an important factor for fungal development, drying represents an effective way of make cereals suitable for storage. The utilization of airflows has proven successful in avoiding OTA contamination by *Penicillium verrucosum*, and this may be extrapolated for different fungi (Annexes A and B; Palumbo et al. 2020b).

As an example, the drying process is commonly applied to maize grain to guarantee a safe storage. If the moisture content of the maize at harvest is not low enough for safe storage, then it must be dried either naturally, with ambient air forced through the storage bin, or artificially in a heated air dryer. Many technologies as well as different temperature x time combinations can be applied for artificial drying. In general, dryers using heated air, with the possibility to change air temperature and flux speed according to the grain batch, are the most commonly used for drying maize in Europe. In general, the product should be dried to less than 14% to be safely stored. Furthermore, a prompt reduction of grain humidity, in combination with lower moisture content (<13%), is suggested when *A. flavus* is present.

Drying is also applied for some grape derived products, like currant, raisin or dessert wines. The drying period duration changes a lot in case of natural or artificial drying conditions, always shorter in the latter. Because *A. carbonarius* is the crucial fungus of interest, the drying period deserves risk of OTA increase, being the fungus more efficient in toxin production with a_w compatible with the early drying period, till when a_w is above 0.93. Even if artificial drying should be always suggested, some products are anyway naturally dried; attention to OTA risk should be paid in this case.

Cleaning and sorting

Susceptibility of the crop to fungal colonization is not only dependent on temperature and a_w, but also on nutrient availability. Harvesting, transportation, drying, and handling of crops generate broken product, foreign material, weed seeds, and other fine materials such as dirt and debris which may act as potential source of mycotoxin contamination. For this reason, segregation of broken, damaged or infected grains, kernels, or lots is also a critical step.

Cleaning and sorting intend to screen cereal grains and eliminate undesirable materials, both other than the cereal itself and damaged kernels or grains that do not met the required specifications.

As examples, removing broken kernels and smaller parts from maize reduced DON and ZEN contamination by around 70–80%. For maize, it has been also demonstrated that intact kernels contained about 10 times less fumonisins than broken corn kernels or smaller parts. The effect of cleaning and sorting in wheat samples leads to lower levels of contamination, with greater impact being caused by mechanical gravity separation. Besides that, on oat experiments, the effect of cleaning step on reduction of T-2 and HT-2 was not significant. One effective way to reduce mycotoxins contaminations might be the inclusion of colour sorting in the cleaning step. In fact, this method was proven to be successful in reducing mycotoxins risks, since it provides an appropriate segregation of *Fusarium* species contaminated grains.

Several physical processes are applied to feed commodities in order to mitigate targeted mycotoxins (e.g., sorting, sieving, flotation, density segregation, etc.). Sorting can be achieved by several automated removing systems. Originally, grain sorting machines were based on particle weight and size grains and sorting was obtained using centrifugation force and flotation in air flow. Contemporary grain sorters are mainly based on optical sensors. Grain sorting using UV light illumination for AF reduction is widely used.

The efficacy of sorting is strongly related to target fungi and mycotoxin. It is commonly confirmed that kernels infected by *F. graminearum* show visible symptoms, like bleaching and shrivelled kernels. It is therefore logic that sorting based on optical sensors contributes significantly to DON reduction. On the other hand, in *F. verticillioides* natural infection relationship between visible symptoms and FUM contamination was never demonstrated; no impact of optical sorting on FBs contamination is expected.

3.1.1.3. Post-harvest

Storage

The essential parameters to control storage are temperature, a_w, and time. Before any processing step, and depending on crops, storage of variable length, depending on the product and its destination, occurs.

Products as cereals are stored for long periods, after the initial management steps. Grain a_w, together with temperature and relative humidity conditions during storage, are the main driven forces for safe storage (Annexes B; Palumbo et al., 2020b). In these cases, drying, sorting, and storage are the critical steps, since later processing stages will commonly not mitigate the occurrence of mycotoxins. Other crop products may be included in this set of commodities, as dried vine fruits (Gonçalves et al., 2020) or spices (Annex E).

Products with high a_w, that may not be dried to safe conditions without changing their nature should be stored for shorter periods of time, having in mind that their natural a_w content will favour fungal development. These products should be processed as soon as possible, or stored in modified conditions (e.g., refrigeration). Examples of this kind of products are fruits, and grapes for wine.

Processing

Food processing can interfere with the stability of mycotoxins present in a certain matrix. Parameters used during processing may reduce, or sporadically increase concentrations, by the different distribution of mycotoxins among the obtained fractions. Control of temperature, time and size of the samples is essential when studying the fate of mycotoxins under process conditions. Reduction of mycotoxin content during processing may be achieved due to physical, (bio) chemical or biological interactions.

It has been clearly reported in the literature that food processing can reduce mycotoxin exposure by (i) destroying or eliminating mycotoxins, by (ii) transforming them into less toxic derivatives, by (iii) adsorbing mycotoxins to solid surfaces or by (iv) reducing their bioavailability due to chemical attachment to food matrix structures. Reduction of mycotoxin contamination was documented for milling; brewing; fermentation;

cooking; baking; frying; roasting; flaking; alkaline cooking; nixtamalization; and extrusion. Most of the food processes have variable effects on mycotoxins, with those that utilize high temperatures having the greatest effects. In general, processes reduce mycotoxin concentrations significantly, but complete elimination cannot be achieved with processing commonly applied, therefore keeping products according to consumer expectation.

Although generally processing will contribute to a reduction in the final mycotoxin content (Figure 16 and (Gonçalves et al., 2019; Gonçalves et al., 2020; Palumbo et al., 2020b); **Annex A-D**), the relative reduction in the content is very dependent on the type and sanitary conditions of the raw material, as well as on the technology and operation conditions employed while processing.

3.2. Biosynthesis of modified mycotoxins

Mycotoxin mixtures may have different origins in the field. Different mycotoxins can actually be produced by the same fungi on the crop, or different fungi may infect the same crops in the field leading to the co-occurrence of multiple parental mycotoxins. As previously described in this report (section 3.1), many environmental, ecological, and agronomic factors may affect the relative abundance of co-occurring mycotoxins in the contaminated crops, which may vary from year to year.

However, the number and diversity of potentially co-occurring mycotoxins is even more increased by plant metabolism. Indeed, the contamination of food by mycotoxins is due to host–guest infectious processes, wherein mycotoxins may act as pathogenicity or virulence factors (Hof, 2008). Therefore, plants have developed effective detoxifying systems to counteract fungal infection.

As for other xenobiotics, plants can alter the structures of mycotoxins through their defence mechanisms, via three main phases: chemical modification (phase I and II metabolism) and compartmentalization (phase III metabolism) (Berthiller et al., 2013).

Overall, these reactions may reduce mycotoxin phycotoxicity, and facilitate their sequestration in the plant vacuole or apoplast. Modified forms may also be incorporated into cell wall components (Berthiller et al., 2013).

While hydrophilic xenobiotics already have reactive groups and, therefore, there is no need to undergo phase I metabolism, this mainly occur for introducing on the lipophilic backbone of the parent compound, a chemical moiety prone to conjugation. Typical phase I reactions involve hydrolysis, reduction or oxidation, and are catalyzed by esterases, amidases, P450 monooxygenases, and peroxidases. Typical phase I modifications of trichothecenes involve the hydrolysis of acetylated derivatives, e.g., the transformation of T2 into HT2 in plants (Nathanail et al., 2015).

It must be noticed that phase I biotransformation not always reduces the toxicity of the parental compounds, and in some cases the toxicity may even be increased (Berthiller et al., 2013). This is the case, for instance, of α-ZEL formation from ZEN, being the former far more estrogenic than the latter (EFSA, 2016).

Phase II metabolism is aimed at transforming the parent compound in a more polar – and therefore less toxic – metabolite through conjugation, being indeed a detoxification step itself. These reactions occur through the binding of hydrophilic biomolecules (i.e. glucose, glutathione, fatty acids) to the parent mycotoxin or to its phase I metabolite. The major pathways are glycosylation catalyzed by UDP-glycosyltransferases (UGTs) and glutathione conjugation catalyzed by glutathione-S-transferases. Among *Fusarium* modified mycotoxins, DON-3-O-β-D-glucoside (DON-3-Glc) is so far the best documented phase II metabolite of DON, but sulfate-, glutathione-, and cysteine-conjugates were also reported.

The higher polarity of the conjugate metabolite leads to an increase in solubility, and therefore facilitates its phase III elimination or storage (i.e. vacuole, apoplast or cell walls). The overall effect of this process is a reduction of the mycotoxin phytotoxicity (Berthiller et al., 2013). The β-glucosides of *Fusarium* mycotoxins, DON-3-Glc, HT2 toxin-3-Glc, and ZEN-14- Glc, are the most common compounds (EFSA, 2017c, b, a).

It must be underlined that, while the lower toxicity of conjugated mycotoxins *per se* is demonstrated, these compounds can undergo cleavage in the gastrointestinal tract of animals, or along the food processing chain, thus releasing the parent mycotoxins (EFSA, 2017a, b, c, 2018b). Therefore, their formation and co-occurrence with parent compounds must be regarded as a relevant contribution to the overall toxic load.

A number of studies have addressed the biosynthesis of modified mycotoxins, with a focus on the enzymes catalysing the process. The efficiency of the detoxification process of mycotoxins in plants depends indeed on the effectiveness of the enzyme produced by the plant in reaching and acting on the mycotoxin.

In particular, the ability to convert DON to DON-3Glc has been proven to be associated with the plant resistance toward Fusarium Head Blight (FHB), a plant disease which causes severe yield losses in cereal crops (Cirlini et al., 2014; Amarasinghe et al., 2016; Nakagawa et al., 2017). Studies comparing the conversion abilities of DON to DON-3Glc in several wheat lines indicated that low glycosylation ability is related to high susceptibility toward fungal infection and toxin accumulation. Gene expression studies allowed to identify the most effective quantitative trait loci associated with FHB resistance and DON-3-Glc accumulation in wheat, which were

mapped on chromosomes 3B (Fhb1, Qfhs.ndsu3BS) and 5A (Qfhs.ifa-5A) (Buerstmayr et al., 2002; Lemmens et al., 2005; Kluger et al., 2015).

Conjugation of mycotoxins with glucose by UGTs in phase II metabolism is one of the primary plant detoxification mechanism for resisting mycotoxin accumulation (Broekaert et al., 2015a).

The earliest study on the detoxification of DON in *Arabidopsis thaliana*, reported on the ability of UGT73C5 to encode for a UGT that catalyzes the transfer of a glucose moiety from UDP-glucose to the hydroxyl group at C-3 of DON. The authors demonstrated that constitutive overexpression of UGT73C5 in *A. thaliana* led to enhanced tolerance towards DON (Poppenberger et al., 2003).

Moving from this first observation, several studies over years reported on different UGTs able to catalysed mycotoxin glycosylation in plants, as summarised in Table 13. In relation to trichothecene glycosylation, reported UGTs shared a regioselectivity towards C-3-OH position.

More recently, some authors identified in wheat 179 UGT genes that could be responsible for the regulation of resistance mechanisms towards FHB infection and DON accumulation (He et al., 2018).

Table 13: Genes encoding for UGTs reported in the literature for the formation of modified mycotoxins

UGTs encoding genes	Mycotoxin	Glucosides	Reference
UGT73C5 (*A. thaliana*)	DON	DON-3-Glc	(Poppenberger et al., 2003)
UGT73C6 (*A. thaliana*)	ZEN	ZEN-14-Glc	(Poppenberger et al., 2006)
HvUGT13248 (*Hordeum vulgare* subsp. *vulgare*)	HT-2 NIV DON	HT2-3-Glc NIV-3-Glc DON-3-Glc	(Li et al., 2017; Michlmayr et al., 2018; Mandalà et al., 2019)
OsUGT79 (*Oryza sativa*)	HT-2 NIV DON	HT2-3-Glc NIV-3-Glc DON-3-Glc	(Michlmayr et al., 2015; Michlmayr et al., 2018)
OsUGT79 H122A/L123A/Q202L (*Oryza sativa*)	T2 DON	T2-3-Glc DON-3-Glc	(Wetterhorn et al., 2017)
Bradi5g03300 (*Brachypodium distachyon*)	HT-2 DON	HT2-3-Glc DON-3-Glc	(Schweiger et al., 2013; Pasquet et al., 2016; Gatti et al., 2018; Michlmayr et al., 2018)
HvUGT14077 (*Hordeum vulgare* subsp. *vulgare*)	β-ZEL α-ZEL ZEN	β-ZEL-14-Glc α-ZEL-14-Glc ZEN-14-Glc	(Michlmayr et al., 2017)
TaUGT-2B (*Triticum aestivum*)	DON	DON-3-Glc	(Sharma et al., 2018)
TaUGT-3B (*Triticum aestivum*)	DON	DON-3-Glc	(Sharma et al., 2018)

3.2.1. Fusarium modified mycotoxins: trichothecenes

DON-3Glc is largely the most reported and described modified mycotoxin. It is formed in plants by conjugation of a glucose moiety to the C-3 hydroxy group of DON. Since the hydroxyl group in C-3 is responsible for the binding of DON to the ribosome binding pocket, thus inducing ribotoxicity, glycosylation induces a loss of toxicity. In addition, it alters the polarity of the molecule, favouring the exit of the cytosol, due to access to membrane-bound transporters. The conjugated mycotoxin is then stored in the plant vacuole or apoplast or bound to the cell wall (Berthiller et al., 2013).

Among cereals, a higher DON-to-DON-3-Glc conversation rate was observed in spelt, followed by wheat and barley, and at lower extent in corn and rye. No conversion was observed in oats so far. The conversion seems to occur mainly at the germination stage, when glucose level is higher (Maul et al., 2012). In addition, DON-3-Glc formation seems to be affected by the parent toxin level. Higher DON concentrations were reported to alter the efficiency of DON to D3G conversion, thus suggesting a limitation in the glycosylation capacity of the plant. In addition to glycosylation reaction through glycosyltransferases, also the glutathione pathway has been described for DON detoxification in plants, mainly mediated by glutathione-S-transferases. Sulphation has been reported as well, with the formation of DON-3-sulfate and DON-15-sulfate.

Accordingly, besides DON-3-Glc, other modified forms of DON have been reported in cereals, mainly wheat, among them DON-hexitol, DON-S-cysteine, DON-S-cysteinyl-glycine, DON-glutathione, DON-di-hexoside, and DON-malonyl-glucoside. In addition, conjugation products of 15-acetyl-DON have been described as well, i.e. 15-acetyl-DON-3-β-D-glucoside.

The biotransformation occurring to other type B trichothecenes, has been understudied compared to DON. However, several authors reported the formation of FUS-X and NIV glucosyl-conjugates in wheat (Nakagawa et al., 2011; Nakagawa et al., 2013; Yoshinari et al., 2014).

Concerning type A trichothecens, the biotransformation of T2 have been studied in barley and oats (Nathanail et al., 2015), leading to the annotation of a number of putative T2 and HT2 glycosyl- and/or malonyl-conjugates. Notably, the authors described the quick phase I conversion in plants of T2 into HT2 through deacetylation. Simultaneously, the HT2 formed was further metabolized through glycosylation to HT2-3Glc.

3.2.2. Fusarium modified mycotoxins: zearalenone

Biotransformation in plants has been described for the xenoestrogenic mycotoxin ZEN, as well. In particular, it undergoes both phase I and phase II metabolism in plants, with the formation of glucoside- and sulphate-conjugates of ZEN and its phase I metabolites α- and β-ZEL.
Glucosilation may occur at both OH group in the phenolic ring, although the formation of ZEN-16-Glc is sterically unflavoured compared to ZEN-14-Glc (Paris et al., 2014; Michlmayr et al., 2017). Unlikely DON, ZEN undergoes an extensive biotranformation in plants, leading to a plethora of different conjugates originated from the parent compounds and its phase I metabolties, as described by Berthiller et al. (2006) in *A. thaliana* and more recently by Righetti et al. (2016) in durum wheat model system (Berthiller et al., 2006; Righetti et al., 2017).

3.2.3. Fusarium modified mycotoxins: fumonisins

Several modified forms of fumonisins have been described so far (EFSA, 2018b, a), mainly due to the effect of processing along the food and feed production chain. However, the possible formation of fatty acid esters of FB1 (O-fatty acyl FB1) and other fumonisins with variation in fatty acid chain length and position of esterification (3-O-, 5-O- or 10-O-acyl-fumonisins), have been described in maize by Bartok et al (2010, 2013a, 2013b) and Falavigna et al. (2013, 2016) (Bartok et al., 2010; Bartok et al., 2013a; Bartok et al., 2013b; Falavigna et al., 2013; Falavigna et al., 2016).

Differently from what described for modified forms of trichothecenes and zearalenone, these conjugates are formed by the fungus when growing on a plant substrate and not by the plant itself. Besides O-fatty acylfumonisins, the corresponding N-fatty acyl fumonisins were also detectable in low amounts in *F. verticillioides* cultures (Bartok et al., 2013b). These phase II metabolites have been found in maize at very low concentration, but occurrence data are extremely poor to date.

3.2.4. Ochratoxin A modified forms

Little is known so far about the possible biotransformation of OTA in plants. Preliminary studies were performed in plant-cell suspension cultures by Ruhland et al. (1996)(Ruhland et al., 1994). A very quick biotransformation was observed in carrots, tomatoes, and corn cell cultures, while longer time was required for degradation in wheat, barley and potatoes. Among metabolites, OT-α, 4S-OH-OTA, 4R-OH-OTA, OH-OTA-β-Glc, and OTA methyl ester, were described.

A follow-up study was performed by Wang et al. (2014) in *A. thaliana* model system, showing that glutathione synthase, glutathione-S-transferase and glutathione peroxidase, significantly increased in tissues treated with OTA, thus suggesting the activation of a glutathione-based detoxification pathway. However, no further studies on the formation of OTA modified forms in plants have been performed so far (Wang et al., 2014).

3.3. Methods of analysis

The methods of analysis can be divided into two broad categories. Those that are referred to as 'screening tests' which give a qualitative or semi-quantitative results. They are generally based on antibody recognition methods such as ELISA and lateral flow. The former is usually laboratory based whilst the latter can be applied in the field. They are often specific to one mycotoxin such as AF, DON, FB etc and require relatively straight forward sample preparation, in some cases simple aqueous extractions can be performed. In more recent times some multiplex rapid screening test have entered the commercial markets allowing up to 5 or 6 mycotoxins to be detect simultaneously. The most promising techniques in terms of multiplexing are based on biosensor platforms but as yet few have made their way to commercial products. This is in part due to the cost of the technology platforms themselves which can detract from the 'low cost' element needed for screening tests. However, there is a growing number of publications outlining that electrochemical biosensors may provide a low cost multiplex solution in the future. In virtually every case the mycotoxins covered by immunoassay based screening tests are for mycotoxins that are regulated. Thus clearly it can be seen that regulations drive testing.

The second tier of testing is often referred to as 'confirmatory analysis'. The methodologies are virtually all based on some form of physio-chemical platform. There are a small number of publications that outline the use of capillary electrophoresis but it is not a widely used technique either in the research or routine testing environments. The most widely used method for a long time was High Performance Liquid Chromatography (HPLC). Quite a number of variations to this methodology in terms of sample preparation, mobile phases etc have been published. HPLC was the method of choice for many to produce quantitative analysis for single or all regulated mycotoxins present in feed and food. In some cases, this is still the method of choice, particularly in the laboratories in Developing Countries where the lack of more sophisticated physio-chemical equipment limits choice. In contract, in the Developed World the most frequently used technology is liquid chromatography coupled to mass spectrometry (LC-MS). The liquid phase is used to separate the compounds of interest from interfering molecules while the mass spectrometry portion of the methodology is used to identify and quantify the mycotoxins themselves. There are many variations in terms of the types of sample preparation methods applied, the forms of liquid chromatography used and mass detectors employed. There is a large range of differences in method performance in terms of speed, sensitivity, specificity and robustness across these methods. There is also a large number of mycotoxins that can be detected and quantified on the LC-MS platforms. Some authors report over 400 compounds being measured simultaneously. Not only the regulated mycotoxins can be measured but many other hundreds of other known mycotoxins for which analytical standards are available. Where sample contain mixtures of mycotoxins (which is a considerable number) the application of such testing methods is vital to determine accurately the range and concentrations of mycotoxins present. These methods can be very expensive to perform, not only because of the cost of the equipment but also the costs associated with sample preparation, analytical standards and highly trained staff to conduct the analysis. Yet in terms of fully understanding animal and human exposure to the board family of mycotoxins such methodologies should be considered as vital (Annex F).

3.4.   Occurrence and co-occurrence of mycotoxins in different crops

The thorough analysis of (co-)occurrence data provided 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 intended for publication in the scientific literature (Palumbo et al., 2020a). In brief, 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 respectively wheat (34 %), maize (28 %), barley (10 %), oat (9 %) and rice (6 %). 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). Mycotoxins were mainly reported in wheat and maize showing the highest concentrations of FBs, DON, AFs, and ZEN. The maximum concentrations of FB1+FB2 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.

All data, plots, fitting distribution parameters and R-code produced regarding mycotoxin occurrence/co-occurrence are available online on MYCHIF repository and EFSA knowledge junction (10.5281/zenodo.3615174).

3.5. Toxicokinetic and Toxicity (toxicodynamic)

3.5.1. Toxicokinetic and Toxicity and databases

Toxicity and toxicodynamic data for selected mycotoxins (further information on the mycotoxins analysed in this work are detailed in Section 1) are reported in specific databases: toxicokinetic database (*in vitro* and *in vivo*), and *in vivo* toxicity database.

The *in vitro* toxicokinetic database was built from 48 peer reviewed articles selected from the ELS wherein data on buffalo (1 paper), bovine (3 papers), carp (1 paper), chicken (3 papers), Chinese hamster (1 paper), dog (1 paper), duck (1 paper), goat (1 paper), human (22 papers), pig (5 papers), quail (1 paper), trout (2 papers), rat (5 papers) and turkey (1 paper) were included. The inclusion criteria used to include papers are detailed in the Section 2.3.2.1.

The *in vivo* toxicokinetic database was built from 109 peer reviewed articles including TK studies on chicken (18 papers), buffalo (3 papers), cow (10 papers), donkey (1 paper), duck (1 paper), sheep (3 papers), goat (2 papers), horse (1 paper), human (8 papers), fish (4 papers), mouse (8 papers), monkey (2 papers), pig (23 papers), rabbit (1 paper), rats (24 papers) and turkey (5 papers).

Finally, *in vivo* toxicity database was built from an ELS covering the years 2010-2017 with a total of 53 papers were included in the database. The focus went on chickens (38 papers) and pigs (15). The mycotoxins addressed were DON (7), ZEN (8), FBs (5) and AFs (33) with dose response data. Moreover, an additional literature search for the period 1976-2010 before 2010 was performed because of the limited data available for the period 2010-2017 and a total of 62 papers including data on chicken (26 papers), mouse (5 papers), fish (2 papers), rat (5 papers), pig (13 papers), hamster (1 paper), quail (3 papers), turkey (5 papers), rabbit (1 paper) and lamb (1 paper) were collected. A review paper is in preparation with provisional title "Overview of the European Food Safety Authority guidelines on the adverse effects of mycotoxins and their derivatives forms on farm animals".

As supported by several reports, humans and animals are generally exposed to several co-occurring mycotoxins. The toxicity of combinations cannot be predicted based on the toxicity of individual mycotoxins, but only few studies analysed the combination of mycotoxins *in vivo* (Grenier and Oswald, 2011) and *in vitro* (Alassane-Kpembi et al., 2017). Therefore, the lack of studies dealing with combined toxicity of multiple mycotoxins suggested the need for a review providing a summary of the current knowledge, underling the weakness of some studies, and guidelines for a proper planning of future studies on toxicity of mycotoxin mixtures *in vivo*. In addition, this review should collect the toxicity values with the doses tested and the adverse effects observed by animal species.

3.5.2. Toxicokinetic *in vivo*

Data collection and subsequent analysis of *in vivo* toxicokinetic data provided the basis to for a review on the topic to be published in the scientific literature. Such a review covers the analysis and comparison of kinetic parameters for single and multiple mycotoxins in different animal species (Gkrillas et al., submitted). In brief, the review highlighted the complexity of studying the toxicokinetic 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 toxicokinetic 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, pigs in respect to rats and calculation of uncertainty factors on interspecies toxicokinetic variability was performed. 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.

Mycotoxins dosage, exposure pathway, interspecies and intraspecies differences were identified among the most important parameters that may influence the toxicokinetics of mixtures. As a general remark, a limited availability of scientific papers on mixtures in comparison with the single compounds was described. 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 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.

3.5.3. Toxicodynamic: an example of an *in silico* structural approach

The thorough analysis of toxicity data allowed the design of a computational-driven structure-based toxicodynamic study, intended for the publication in a scientific journal, comparing the estrogenicity of ZEN, its metabolites and the emerging mycotoxin alternariol (AOH) in human and trout. Inter-species differences in their toxicokinetic and toxicodynamic may occur depending on evolution of taxa-specific traits. As a proof of principle, this manuscript investigates the comparative toxicodynamic of ZEN, its metabolites (alpha-zearalenol and beta-zearalenol), and alternariol (which is an emerging threat in fishing farming) with regards to estrogenicity in humans and rainbow trout. An *in silico* structural approach based on docking simulation, pharmacophore modelling and molecular dynamics was applied, and computational results were analyzed in comparison with available experimental data. The differences of estrogenicity among species of ZEN and its metabolites have been structurally explained. Also, the low estrogenicity of alternariol in trout has been characterized here for the first time (Dellafiora et al., 2020).

Overall, the work described the usability of *in silico* modelling to better understand the toxicodynamics of mycotoxins focusing on the inter-species variability. Such approaches will provide a useful analysis to complement the characterization of inter-species mycotoxins toxicity by: i) understanding the structural basis of mycotoxins toxicity; ii) predicting the capacity to differentially trigger biological and toxicological stimuli; iii) driving future analysis through the evidence-based prioritization of compounds, endpoints and species of interest to risk assessment; iv) integrating toxicokinetic data for a more comprehensive understanding of mycotoxins toxicity; v) supporting biologically-based interpretation of toxicological data to improve extrapolation between species and the assessment of human relevance.

3.5.4. Coarse-grained calculation of individual kinetic parameters for selected mycotoxins

A list of selected mycotoxins underwent a coarse-grained and qualitative calculation of individual kinetic parameters using the SwissADME tool (Daina et al., 2017). In particular, passive human gastrointestinal (HGI) absorption, blood-brain barrier (BBB) permeation, glycoprotein (Pgp)-mediated permeability and possible inhibitory activity *versus* a number of cytochromes P450 (i.e. CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4) were calculated. The calculation of passive HGI absorption and Pgp-mediated permeability is based on the BOILED-Egg model (Daina and Zoete, 2016), which defines favourable and unfavourable zones in the hydrophobicity (expressed as logP o/w) versus polar surface area physicochemical space for passive diffusion through both physiological barriers. The calculation of Pgp-mediated permeability and possible inhibitory activity *versus* a cytochromes P450 relies on support vector machine (SVM) algorithm, properly feed with meticulously cleansed large data-sets of known substrates/non-substrates or inhibitors/non-inhibitors, as reported by Daina and co-workers (Daina et al., 2017). The results collected are summarized in Table 14.

Table 14: Individual TK parameters for selected mycotoxins

Mycotoxin	Transport			CYP450 inhibition				
	GI absorption	BBB permeant	Pgp substrate	CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4
Aflatoxins								
AFB1	High	Yes	No	No	Yes	No	Yes	Yes

AFB2	High	Yes	No	No	Yes	No	Yes	Yes
AFG1	High	No	No	No	Yes	No	No	No
AFG2	High	No	No	No	Yes	No	Yes	No
Alternaria toxins								
AOH	High	No	No	Yes	No	No	Yes	No
AME	High	No	No	Yes	No	No	No	No
TEA	High	No	No	No	No	No	No	No
ATX-I	High	No	Yes	Yes	No	No	No	No
ATX-II	High	No	No	Yes	No	No	Yes	No
Enniatins								
ENNA	Low	No	Yes	No	Yes	No	No	No
ENNA1	Low	No	Yes	No	Yes	No	No	No
ENNB	High	No	Yes	No	No	No	No	No
ENNB1	Low	No	Yes	No	No	No	No	No
BEA	Low	No	Yes	No	No	No	No	No
Fumonisins								
FB1	Low	No	No	No	No	No	No	No
FB2	Low	No	No	No	No	No	No	No
FB3	Low	No	No	No	No	No	No	No
FB4	Low	No	No	No	No	No	No	No
Trichothecenes								
15-ADON	High	No	Yes	No	No	No	No	No
3-ADON	High	No	Yes	No	No	No	No	No
DON	High	No	Yes	No	No	No	No	No
DAS	High	No	No	No	No	No	No	No
NEO	High	No	Yes	No	No	No	No	No
NIV	Low	No	Yes	No	No	No	No	No
T2	High	No	No	No	No	No	No	Yes
HT2	High	No	No	No	No	No	No	Yes
FUS-X	High	No	Yes	No	No	No	No	No
Zearalenone and metabolites								
ZEN	High	No	No	No	No	Yes	No	Yes
alpha-ZEL	High	No	No	No	No	No	No	No
beta-ZEL	High	No	No	No	No	No	No	No
ZAN	High	No	No	No	No	No	No	Yes
Other mycotoxins								
CIT	High	No	No	No	No	No	No	No
CPA	High	Yes	Yes	Yes	No	No	Yes	No
OTA	High	No	No	No	No	Yes	No	Yes
PAT	High	No	No	No	No	No	No	No
STM	High	Yes	No	Yes	Yes	Yes	Yes	Yes

The results collected highlighted the passive GI permeability of the all set of mycotoxins considered, while CPA, STM, AFB1 and AFB2 were also described able to cross BBB. In addition, ATX-I, ENNA, ENNA1, ENNB, ENNB1, BEA, 15-ADON, 3-ADON, DON, NEO, NIV, FUS-X, CPA were described as likely substrate of P-gp, which is a pivotal actor in xenobiotics disposition and distribution to organs (Couture et al., 2006). Many mycotoxins were also computed as possible CYPs inhibitor. Specifically, STM might inhibit all the isoforms considered in the analysis. Taking into account the possible multiple and simultaneous exposure to some of those mycotoxins (vide infra), the results presented here described the likely existence of a complex network of mutual influence on TK parameters with consequences on both metabolisms and transport.

3.6. Biomarkers

Specific molecular markers, biomarkers (BM), may be used to measure the extent of the exposure to a toxic substance (Groopman et al., 1994). Biomarkers may be measured in body fluids or tissues as parent molecule or as a metabolite, anyhow, to be functional, a biomarker shall correlate with the dietary intake, be available with a suitable persistence, and be measurable in the specimen with suitable specificity and precision. Biotransformation pathway may differ depending on the contaminant so that the biomarker measured may be found in urine, blood, breast milk and, more rarely, in other fluids or tissues such as sputum and faeces or biopsies tissues of lung, liver and brain.

Biomarkers have been divided in three categories: i) **biomarkers of exposure**, which indicate that the exposure to a particular contaminant has taken place in a certain extent; ii) **biomarkers of effect**, which are indicators of the biological response of the exposure; iii) **biomarkers of susceptibility**, which act as indicators of the intrinsic sensitivity of individuals to the toxic agent (Timbrell, 1988; Groopman et al., 1994).

The measurement of biomarkers of exposure represent an exceptional method either to directly confirm an exposure event or to substantiate the relevance or applicability of results derived from probabilistic exposure studies (Kroes et al., 2002). However, biomarkers represent a measure of the overall exposure not discriminating between different sources (i.e. food or air) (Aitio and Kallio, 1999). This is not a limit for some chemical contaminants, such as mycotoxins, whose exposure is mainly due to food ingestion. Anyway, biomarkers represent a complementary method to the classic estimates of exposure based on food consumption and concentration levels, and they have indubitable advantages for measuring the exposure over time, for estimating the exposure directly (not relying on models and uncertainty assumptions) and for assessing individual estimates (especially for specific subpopulations e.g. vegetarians, celiac, etc). Moreover, biomarkers measurements is not subject to drawbacks for the heterogeneity of food contamination, nor to variability in food processing and cooking (Wild and Gong, 2010), both critical in the measurement of mycotoxins contamination in food. Certainly, to be reliable and to provide accurate estimate of exposure, biomarkers must be validated. The validation process is complex and implies a parallel experimental design to confirm the connection between the marker and the exposure (Groopman et al., 1994).

3.6.1. Biomarkers of exposure and effects in animals

Biomarker measurements are a useful tool to be used to complete the information about exposure, metabolism, toxicology and the carry-over rates of the different parent compounds or metabolites in the context of animal exposure to hazards. Specific studies, focused on the fate of the mycotoxin in different tissues and organs (e.g. cardiac muscle, kidney, liver) or biological fluids (blood, urine or milk), have been performed so far to put evidence on the big potential of the BM studies to depict exposure scenarios.

The focus of the data review managed in this project was driven by the rationale to have included all the studies where BM were used for assessing animal exposure, for measuring the carryover and for driving knowledge for toxicological study and understanding the toxicodynamic. The selection criteria foresaw to include the kind of toxicological studies that were planned to study toxicokinetic (TK) or toxicodynamic (TD) parameters, in which the outputs from the BM perspective were considered as valuable information.

Regardless of the study, a fundamental point highlighted was the availability of validated analytical methods able to produce sound and transferable data. On this purpose the gathered information provided a systematic review of the available data on BM measurement also giving evidence on the reliability of the methods, in terms of performance characteristics such as LODs and LOQs and validation.

The final number of references about the animal studies after the selection criteria summed up 65 articles; those addressed to multi-biomarker measurements where the 40%, the remaining 60% focused on a single mycotoxin, most often the parent compound.

Table 15 summarizes the number of records registered for each mycotoxin. The number of references and the type of study are also reported. As it is shown, the number of publications on aflatoxins is the majority followed by DON, OTA, and ZEN. Figure 17 synthesizes the distribution between the two groups of studies (*BM of*

exposure and TK study with data on biomarkers) and the number of references that populated the animal dataset.

Table 15: Summary of the mycotoxins, number of records, number of references and the type of study present in the dataset of animal studies on biomarkers

Mycotoxin	N# records	N# of references	Type of study
AFs	243	22	13 BM of exposure 9 Tox studies with data on BM
OTA	87	11	9 BM of exposure 2 Tox studies with data on BM
DON	213	12	6 BM of exposure 6 Tox studies with data on BM
T2/HT2	6	1	Tox studies with data on BM
NIV	32	1	Tox studies with data on BM
FBs	108	3	1 BM of exposure 2 Tox studies with data on BM
ZEN	306	9	BM of exposure
BEA	10	1	Tox studies with data on BM
ENNs	22	4	Tox studies with data on BM
STC	6	1	BM of exposure
	1033	65 references in total	39 studies BM of exposure 26 Tox studies with data on BM
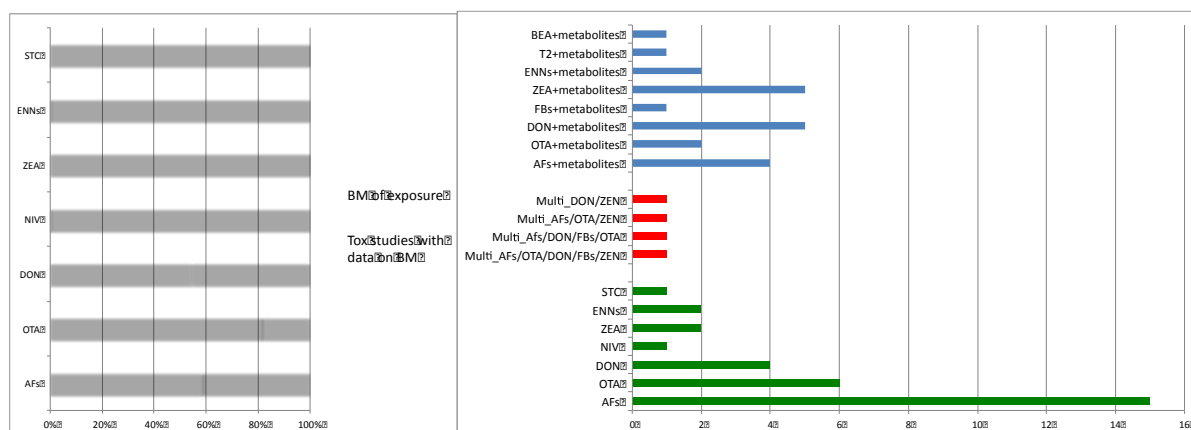
Figure 17: Distribution of the type of studies (BM of exposure and Toxicokinetic study with data on biomarkers) and the number of references for single and multi-mycotoxin study

Annex K reports supplementary material as tables (K1-K6) which summarise the single biomarker history for AFs (K1), OTA (K2), DON (K3), FBs (K4), ZEN (K5), NIV (K6), and ENNs, BEA and STC (K7). Supplementing tables list the substrate, the kind of study (either for exposure or a toxicological study), the animal (sample type), the analytical technique used and the range of levels found (including the LOQ or LOD value). Moreover, supplementing tables K8-K12 summarizes the information from the animal perspective: K8 for poultry, K9 for suidae, K10 for bovidae, K11 for rat and mice, and K12 for horse and dog. For each group of animals clustered, supplementing table lists the biomarker, the sample description, the substrate, the range of values, the analytical method and the conclusions.

3.6.1.1. Aflatoxins

The largest number of publications (N=22) examines AFs. This is understandable due to the toxicological concern of AFB1 and for the issues that still remain unsolved about the definition of the excretion rate of all the different metabolites. The majority of the studies is about the BM measurement for exposure assessment. In fact, AFM1 in animal milk is the most represented biomarker (14 references). However, AFB1 in faeces, rumen fluid, milk, and plasma and AF-adducts (albumin, lysine or guanine) are also considered both in toxicological studies and in exposure assessment. Seven out of the reported publications dealing with a multi-biomarker study where the assessment of AFB1 is in combination with one or more metabolites or with other mycotoxins (most often OTA).

As regards the BM analytical technique, ELISA methods for AFM1 are used in the majority of publications, lamentably, often without indicating the LOD/LOQ of the method that represents a crucial information to assess the strength of the analytical results. HPLC-FLD and LC-MS/MS techniques are also reported almost in combination with purification step, which is necessary to reach a suitable sensitivity.

Table K1 in annex K shows AFs and each AF BM reported in the different substrates of the associated animal.

3.6.1.2. Ochratoxin A

OTA is present in 11 publications of which 9 are about the BM presence in exposure studies whilst 2 are about toxicological studies. OTA has been studied in a wide range of substrates (see table K2). Either for its metabolism and distribution this toxin can be found in kidney, liver, bile, rumen fluid, faeces, plasma and urine. OTα, the hydrolysed form of OTA, is the only metabolite that is reported in the present dataset. OTA is present in a number of multi-biomarker publications especially in combination with AFs. The majority (82%) of the scrutinized references have been reported OTA in exposure assessment studies. OTA analyses are mainly carried out by HPLC-FLD or LC-MS/MS with good sensitivity and few are the authors who did not reported analytical LOD or LOQ.

Table K2 in annex K shows the list of substrates and the range of levels found for OTA and OTα.

3.6.1.3. Deoxynivalenol

DON and its numerous metabolites are present in 12 publications, 6 about the biomarker presence in exposure studies and 6 in toxicological studies. Urine, serum and plasma are the most common substrates for the biomonitoring studies of DON, nevertheless there have been publications about the presence of DON and its metabolites in bile, eggs, excreta and faeces (see table K3). DON has a high number of metabolites, de-epoxidised form (DOM1), gucuronidizated metabolites (3 and 15 DON glucuronide forms), and sulfonates forms that are measured in various substrates. Due to the complex metabolism, DON is often measured in combination with its metabolites, and also with other toxins, which with it may co-occur in the exposure.

Table K3 shows the list of substrates and the range of levels found for DON and DON metabolites.

3.6.1.4. Fumonisins

Quite few publications were found in literature. Hydroxylated derivatives of FB1 (HFB1 and pHFB1 a/b) are the BM reported in animal studies for fumonisin B1 and summarized in table K4. FBs are known to have a rapid distribution and excretion rates; the BM were detected in faeces and urine. Between the BM of exposure, the ratio of Sa and So (Sa/So rates) is a well-known parameter used for FBs exposure and Masching (Masching et al., 2016) reports values of Sa, So and Sa/So rates (table K4).

Due to the recurrent occurrence in maize, FBs are often studied in multi-biomarker studies, where the presence of FBs metabolites and other toxins are measured in faeces and urines (Gambacorta et al., 2016; Pantaya et al., 2016).

3.6.1.5. Zearalenone

ZEN is studied in combination with a high number of metabolites; it is present in 9 publications of which 7 report the entire metabolites group. ZEN, ZAN, α- and β-ZEL, α- and β-ZAL unconjugated, glucuronide and sulphate forms have been quantified in different substrates, urine and faeces being the preferable excretion route (table L5). However, there have been publications about the presence of ZEN and its metabolites also in bile, cardiac muscle, kidney, liver, lung, muscle, ovary, spleen, uterus and plasma (Winkler et al., 2014; Gajęcka et al., 2016; Ueberschär et al., 2016). Due to the complex metabolism, ZEN is always measured in combination with its metabolites and in combination with other *Fusarium* toxins (Winkler et al., 2014) or other toxins (Gambacorta et al., 2016).

Table K5 shows the list of substrates and the range of levels found for ZEN as biomarker.

3.6.1.6. Nivalenol and T2 toxin

Two references, one for NIV and one for T2, have been selected in this database (Sun et al., 2014; Kongkapan et al., 2016a). Both are toxicological studies carried out to study metabolic pathways of these molecules (see table K6).

Of the possible metabolites of T2, HT2 and T2triol are those monitored in plasma and urine. The study has measured a positive value of the metabolites in urine, excepting for HT2. Sun (Sun et al., 2014) studied the fate of the toxin in different substrate. Colon, heart, kidney, liver, muscle and plasma reported a positive measurement of NIV, but no metabolites were reported.

Table K6 shows the list of substrates and the range of levels found for NIV and T2 as BM.

3.6.1.7. Enniatins, beauvericin, sterigmatocystin

Six references, 4 for ENNs, 1 for BEA and one for STC, have been selected in this database (see table K7). All the references regard toxicological studies carried out to learn about metabolic pathways of these molecules.

Only parent molecules have been measured for these 3 mycotoxins; in fact, no phase II metabolites have been reported for these molecules yet. Fushimi (Fushimi et al., 2014) investigated STC in urine only. ENNs and BEA were monitored in brain, cervical cancer tumor, colon, fat, kidney, faeces, liver, muscle, plasma, serum and urine (table K7).

3.6.1.8. Multi-mycotoxin multi-biomarker

The multi-mycotoxin studies collected comprised all kind of publications showing either study of a single mycotoxin and its metabolites or those studies where a group of 2 or more mycotoxins (with or without metabolites) were simultaneously monitored in different substrates. In the animal dataset the majority of the studies regarded the analysis of the parent toxin with its metabolites, while only 4 publications analysed a group of different mycotoxins.

In Figure 17 the histograms representing the number of studies for each mycotoxin are showed.

3.6.2. Biomarkers of exposure in animal species

Mycotoxins are natural contaminants ubiquitous in agricultural commodities. Cereal and cereal products for animal feeding are regulated with two legislative acts: the Directive EC/32/2002 (European Parliament, 2002), which sets maximum levels for AFB1 and in feed and feed products intended for farming animals, and the Recommendation EC/576/2006 (European Commission, 2006) which establishes guidance values to be applied to verify the acceptability of compound feed and cereal and cereal products for animal feeding. Occurrence data for all the mentioned mycotoxins in feed and feed products are available and data is produced through a continuous monitoring programme in EU Member States.

Five groups of animal species were categorised and clustered: 1) poultry (chicken, hens, and turkeys), 2) bovidae (cow, cattle, buffalo, sheep and goat); 3) suidaes (pigs and wild boars); 4) rat and mice; and 5) other animals (dog and horses).

All the selected studies listed in the dataset were taken into consideration to gather information on the BM presence tracing the parent molecule and the metabolite into the different substrates.

3.6.2.1. Poultry

Chickens and turkeys have been clustered in this group. As it is shown in Figure 18, among all the publications, the most numerous deal with broilers, with DON as the most common mycotoxin studied, while excreta and liver are the most common analysed substrate.

Table K8 sets out data about poultry listing the BM, sample and study description, substrate, range of values and analytical method in each of the reported reference.
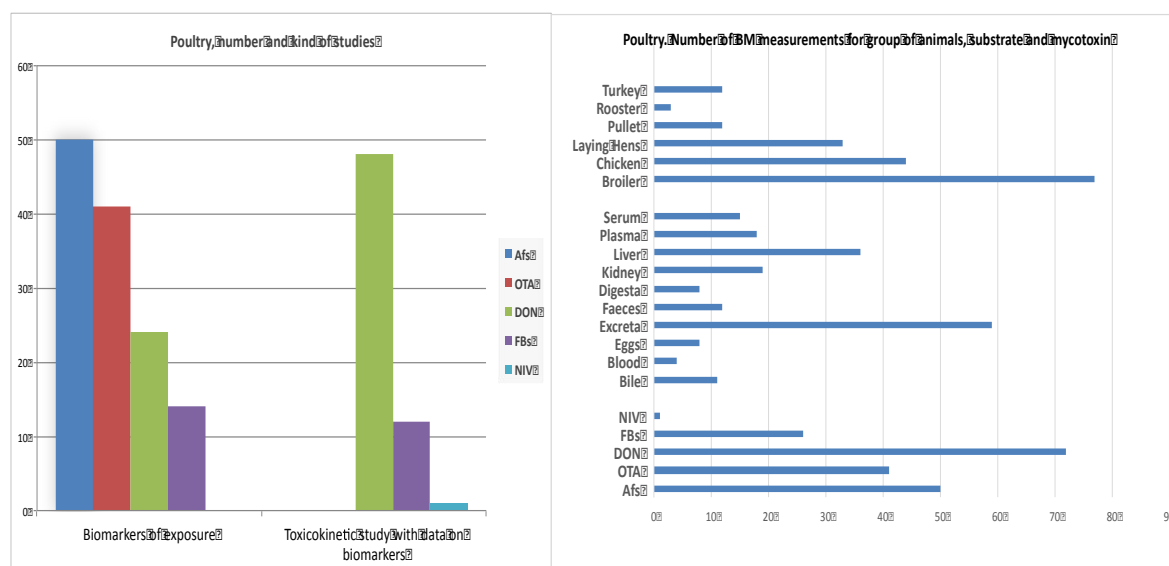
Figure 18: Distribution of biomonitoring and toxicokinetic studies in poultry reporting markers of exposure and the number of BM measurements for poultry species, organs and mycotoxin congeners

Aflatoxin

In poultry, AFB1 and AFM1 measurements in different substrates were used to investigate the effects of lactic acid bacteria on growth performance, and AFs metabolism (Liu et al., 2017) or to check the efficacy of grape seed proanthocyanidin extract (a power antioxidant) in the detoxification of AFB1 in broilers (Rajput et al., 2017). Both the lactic acid bacteria and the supplementation of grape seed proanthocyanidin extract were shown to be effective in the detoxification of AFB1, reducing AFB1 residues. On the other hand, Yaser (2017) explored the efficacy of humic acid as an AFB1 binder in broiler chickens exposed to a AFs-contaminated feed. Humic acid showed protective effects against liver damage and some of the hematological and serum biochemical changes associated with aflatoxin toxicity (Yaser et al., 2017).

Ochratoxin A

Bile (Armorini et al., 2015), liver and kidney (Joo et al., 2013) have been reported as good substrates for the assessment of exposure to OTA. Armorini (Armorini et al., 2015) and Joo (Joo et al., 2013)have carried out

experimental diet treatments where a good correlation between the ingested OTA and the findings confirmed that OTA in these substrates could be considered a good indicator and a suitable biomarker of exposure.

Joo et al. (2013) and Qu et al. (2017) have verified that the use of mycotoxin deactivators or mixed adsorbent in OTA contaminated diets significantly decreased the OTA accumulation in organs and significantly increasing fecal excretion of OTA and its metabolite OTα (Joo et al., 2013) and reduce adverse effects of ochratoxicosis in broilers (Qu et al., 2017). The carryover in eggs was found to be below the limit of detection (Armorini et al., 2015).

Deoxynivalenol and nivalenol

DON and DON metabolites are the most investigated mycotoxins in poultry. From feeding trials with turkeys, chickens, pullets, and roosters Schwartz-Zimmermann (Schwartz-Zimmermann et al., 2015) found out that in excreta and chyme samples DON3sulfate resulted to be the major DON metabolite in all poultry species studied. Devreese (Devreese et al., 2012) set a model for testing the efficacy of DON detoxifying agents in plasma where concentrations of DON were significantly reduced by the detoxifying agents, without detecting the main metabolite, DOM1 (Devreese et al., 2012). DOM1 was not found in none of the plasma and bile samples tested in the feeding trial conducted by Ebrahem (Ebrahem et al., 2014) whose diet included DON contamination (0.4 and 9.9 mg DON/kg). As regards the carryover rates of DON levels transferred in egg yolk and albumen, Ebrahem found ranges from 0.0 to 0.000016, indicating that very low levels of DON are transferred into eggs so that their contribution to human exposure is considered from very low to insignificant (Ebrahem et al., 2014).

NIV is poorly absorbed orally and it is rapidly eliminated via faeces. NIV was found to be able to penetrate into various tissues: small intestine, kidney, heart, liver and muscle (Kongkapan et al., 2016b). The largest quantity of NIV was found in the small intestine suggesting that NIV in broilers is absorbed from the gastrointestinal tract with low bioavailability and has the ability to diffuse into various tissues (Kongkapan et al., 2016b).

Fumonisins

All studies on FBs concern the testing of different agents for their detoxifying capacity. Commercial carboxylesterase or nosilicate clay platelets were supplied to the animals with FBs contaminated diets. A feeding trial was performed by Masching (Masching et al., 2016) to compare a control group with a group fed with a fumonisin contaminated diet. When the supplemented carboxylesterase (FUMzyme) was introduced in the diet, a significant decreased FB1 levels in excreta and a consequent significantly increase of HFB1 concentrations were registered confirming that carboxylesterase is effective to detoxify FB1 in the digestive tract of turkeys (Masching et al., 2016). The same conclusions were obtained by Grenier et al. (2017) who, by the analysis of the sphingoid base, showed an increase of the Sa/So ratio in the serum and liver of broiler chickens fed with FB-contaminated diet as compared to ones fed with an uncontaminated diet (Grenier et al., 2017). The addition of carboxylesterase (FUMzyme R) significantly reduced this specific increase of the Sa/So ratios in serum and liver and was able to hydrolyze FBs in the gastrointestinal tract of chickens. Likewise, Yuan et al. (2017) concluded that nanosilicate clay platelets are as a safe and effective agent for FB1 detoxification as effectively improved the growth performance and ameliorated FB1 toxicosis (Yuan et al., 2017).

Enniatins

ENNB1 and ENNB measurements were included in a toxicokinetic study by Fraeyman (Fraeyman et al., 2016). Both ENNs were found to be poorly absorbed after oral administration and readily distributed to the tissues. The mean total body clearance was rather high, namely, 6.63 and 7.10 L/h/kg for ENNB1 and B, respectively. The study also evidenced that oxygenation was the major phase I biotransformation pathway for both ENNB1 and B. Neither glucuronide nor sulfate phase II metabolites were detected (Fraeyman et al., 2016).

3.6.2.2. Suidae (pig and wild boar)

Pig and wild boar were included in this group. As it is shown in Figure 19, among all the publications, ZEN is the most popular mycotoxin studied, while urine is the preferred most common analysed substrate.

In Annex K supplementing table K9 reports the biomarker, the sample description, the substrate, the range of values, the analytical method and conclusions for suidae studies are listed.
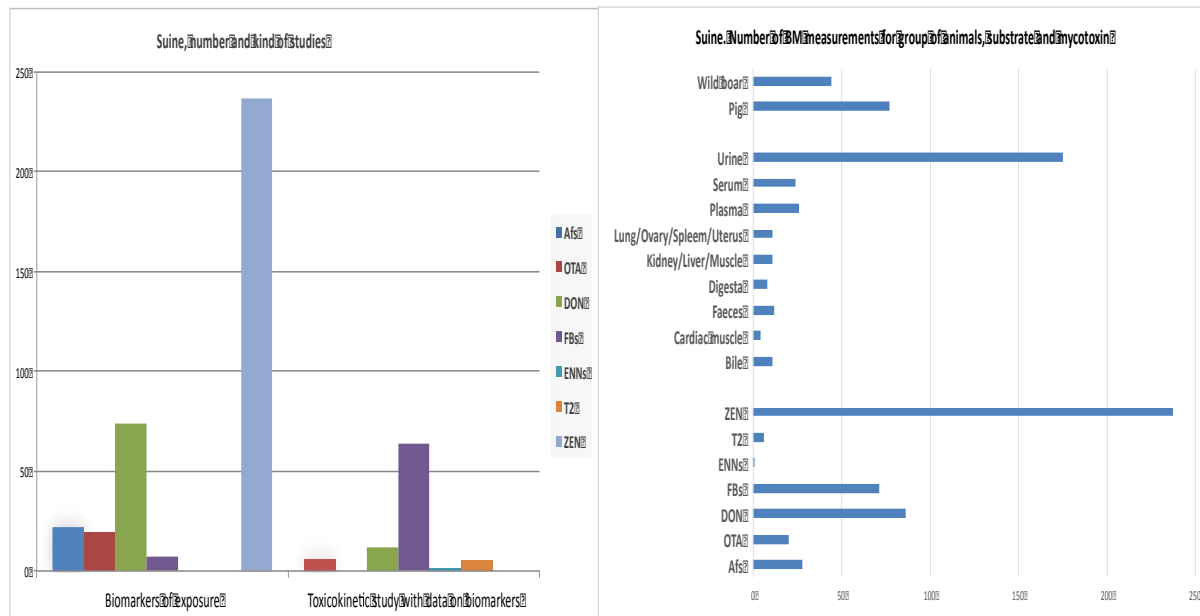
Figure 19: Distribution of biomonitoring and toxicokinetic studies in suidae (pig and wild boar) reporting markers of exposure and the number of BM measurements for suidae species, organs and mycotoxin congeners

Aflatoxins

The articles about AFs are from Di Gregorio (Di Gregorio et al., 2017a; Di Gregorio et al., 2017b) who for one hand set and optimized a method for the determination of AFB1-lys in serum and heparinized swine plasma. This study verified that the use of EDTA did not interfere in AFB1-lys standard detection, but they suggest that EDTA should be avoided during blood collection since it affects the pronase activity in AFB1–albumin adduct digestion and, consequently, causes a reduction in the AFB1-lys levels. Di Gregorio's research also verified that AFB1-lysine is a good AFB1 biomarker for diagnostic purposes and for evaluating the efficacy of chemoprotective interventions of a hydrated sodium calcium aluminosilicate in pigs fed AFB1.

Ochratoxin A

All the publications about OTA in suidae are referred to bio-monitoring studies where the animal exposure was investigated in different countries. This strengthens the reliability to use direct OTA measurements in biofluids to measure OTA levels and possibly prevent the occurrence of ochratoxicosis in animal production, reduce economic losses, and minimize hazards to human health (Kruger et al., 2010).

Pozzo (Pozzo et al., 2010) investigated the different exposure patterns in organic and conventional swine farms in Italy. Despite the fact that all the complementary and complete swine feedstuffs were under the EU recommended guidance values, the OTA contamination of organic feed and serum samples of swine organically fed was found to be significantly higher than that of conventional feed and serum samples (Pozzo et al., 2010).

Four major geographical regions of Brazil were monitored by Kruger (Kruger et al., 2010) with the aim to investigate OTA distribution. OTA distribution in foodstuffs was found to be very heterogeneous. The direct relationship found between the higher concentrations of OTA in serum and the highest concentrations of OTA in food intended for animal consumption strengthens the strategy of measuring the presence of OTA in swine serum samples as an alternative to the feedstuff analyses. Grajewski (Grajewski et al., 2012) compared the OTA presence in kidney of pigs and wild boars during 2006-2007 seasons. Higher level of OTA in the kidneys and serum of wild boars than the levels present in pigs were found in both years (Grajewski et al., 2012). Besides in a study where the impact of the farming system (organic, Label Rouge and conventional) was assessed, the levels of OTA were tested and the liver–muscle pairs of French pigs (muscle and liver) showed OTA liver concentration systematically higher (2.9×OTA) than the muscle (Hort et al., 2018).

Deoxynivalenol, nivalenol and T2 toxin

DON Contamination in grains is common worldwide and pigs are the most susceptible species to this mycotoxin. Deng (Deng et al., 2015) investigated the distribution of DON in plasma, bile and 27 tissues after i.v. administration (250 µg/kg bw and 750 µg/kg bw) and verified that concentrations of DON in tissues differ when pigs are exposed to various dosages and that DON causes lesions in many pig tissues (Deng et al., 2015). The same conclusions were reached by Alizadeh (Alizadeh et al., 2015), who measured the effects of a short-term and comparable DON exposure (0.28 mg/kg bw) on various gut health parameters in pigs. They found out that the DON contaminated diet negatively affected weight gain and induced histomorphological alterations in the duodenum and jejunum. Even after level exposure to DON considered as acceptable in animal feeds, the clinically-relevant changes were measurable in markers of gut health and integrity.

The oral bioavailability of DON3G and its metabolites was assessed by Nagl (Nagl et al., 2014) and seems to be reduced by a factor of up to 2 compared with DON. The authors carried out oral and intravenous application of DON and DON3G. The majority of orally administered DON3G was excreted in form of DON, DON15glucuronide, DOM1 and DON3glucuronide, while urinary DON3G accounted for only 2.6%. In faeces, just trace amounts of metabolites were found. When administered intravenously, DON3G was almost exclusively excreted in un-metabolized form via urine. Data indicate that DON3G is nearly completely hydrolyzed in the intestinal tract of pigs, while the toxin seems to be rather stable after systemic absorption (Nagl et al., 2014).

In a DON-controlled feeding study on weanling piglets (Hopton et al., 2012), the DON ingestion was found to be associated with plasma glucose concentration.

DON sulfonate is another one of the DON metabolites that are urinary excreted. It can be degraded back to DON under physiological conditions as it has been put in evidence in the urinary balance experiment carried out by Frobose (Frobose et al., 2017). The feeding trial set by Frobose and co-authors was performed to assess the effect of commercial products of adsorbent clays and the metabisulfite product. They found out that feeding diets contaminated with 4 mg/kg DON to nursery pigs reduced nursery pig growth, primarily via feed intake suppression. The addition of a commercial product of adsorbent clays (blended with preservatives) did not alleviate the DON-associated effects on pig growth nor did reduce DON absorption and urinary excretion compared to pigs fed DON-contaminated diets alone. However, treating DON-contaminated diets with 1.0% sodium metabisulfite product restored feed intake and improved feed efficiency markedly (Frobose et al., 2017). Gambacorta (Gambacorta et al., 2016) experimented the effect of four agricultural by-products to be used as alternatives to commercial binders to reduce the gastrointestinal absorption of a mixture of mycotoxins. Urinary mycotoxin BM were tested in piglets fed with bolus containing 769 µg of FB1, 275 µg of DON, 29 µg of ZEN, 6.5 µg of AFB1 and 6.6 µg of OTA (2.2, 0.8, 0.08, 0.02, and 0.02 µg/g in the daily diet, respectively). The reduction of mycotoxin absorption was up to 69% and 54% for agricultural by-products and commercial binders, respectively. White grape pomace of Malvasia was the most effective material as it reduced significantly urinary mycotoxin biomarker of AFB1 and ZEN, whereas statistically not significant reductions were observed for FB1, DON, and OTA.

Sun and co-workers (Sun et al., 2014) developed a method for the analyses of T2 and HT2 that was successfully applied to toxicokinetics, tissue distribution, and excretion studies of T-2 toxin and its major

metabolites in pigs. Animals were administered with T2 intravenously and the results provided information for evaluating and controlling human exposure to residual T-2 toxin and its major metabolites.

Zearalenone

ZEN and its metabolite αZEL are nearly exclusively conjugated to glucuronic or sulfuric acid in bile and urine of pigs. Ueberschär (Ueberschär et al., 2016) fed a group of sows with ZEN contaminated diets and ZEN and αZEL concentrations were assessed in urine and bile. In bile samples, both mycotoxins increased significantly with the ZEN concentration of the feed. In urine, such a dependency was found only for αZEL. The percentage of the free ZEN and αZEL varied between 1 and 6 %. The proportion of glucuronidated conjugates of ZEN in bile and in urine of sows was >95 % and no sulfated conjugates were present. The relation of glucuronidated to sulfated conjugates of αZEL was 82 to 17 % in bile, whereas in urine the sulfated form of αZEL was predominant to the glucuronidated form (62 % and 33 %, respectively).

All plant ZEN metabolites (ZEN14- and ZEN16- glucoside) and fungal ZEN metabolites (ZEN14S) can be present in feed, therefore, exposing animals to absorption, distribution, metabolism and excretion (ADME) (Binder et al., 2017). After oral administration of ZEN 10 g/kg bw, ZEN14Sulfate 12.5 g/kg bw, ZEN14G 12.5 g/kg bw, ZEN16G 12.5 g/kg bw, Binder measured the biological recovery of ZEN in urine (26% +/- 10%), and the total biological recovery in excreta. ZEN14sulfate, ZEN14ObGlucoside and ZEN16ObGlucoside were neither detected in urine nor in faeces. After ZEN14sulfate supplementation, 19% of the administered dose was recovered in urine. None of the ZEN metabolites were detected in faeces. The total biological recoveries of ZEN14Obglucoside and ZEN16Obglucoside in the form of their metabolites in urine were 19% and 13%, respectively (Binder et al., 2017).

Multi-mycocotoxins

There is only one study in the animal dataset reporting a multi-mycotoxin study for pigs. The efficacy of four agricultural byproducts and two commercial binders to reduce the gastro-intestinal absorption of a mixture of mycotoxins was tested in piglets using urinary mycotoxin BM as indicator of the absorbed mycotoxins. The study demonstrated that grape pomace reduces the gastrointestinal absorption of AFB1, DON, FB1, OTA and ZEN and it can be considered an alternative to commercial products (Gambacorta et al., 2016).

3.6.2.3. Bovidae

Cows, buffalos, goat and sheep were included in this group. As it is shown in Figure 20, among all the publications considered AFs, more specifically AFM1 in cow milk, are strongly represented.

In Annex K supplementing table S10 lists the biomarker, the sample description, the substrate, range of values, the analytical method and conclusions for bovidae studies.
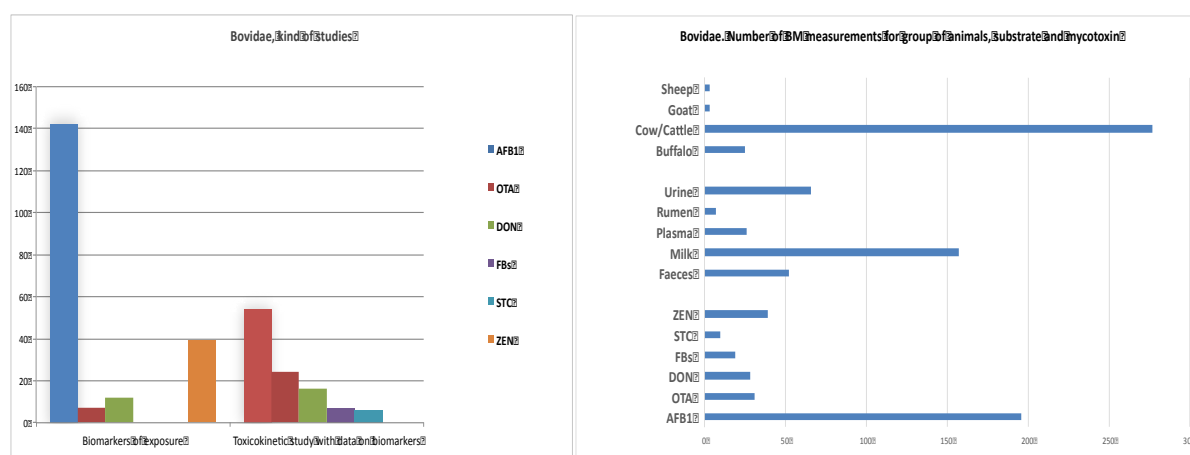
Figure 20: Distribution of biomonitoring and toxicokinetic studies in bovidae reporting markers of exposure and the number of BM measurements for bovidae species, organs and mycotoxin congeners.

Aflatoxins (AFB1 and AFM1)

Mycotoxins metabolites produced by phytopathogenic and spoilage fungi in animal feed as a result of poor storage, can originate in the field and are excreted in milk when dairy animals consume these contaminated feeds, posing a public health risk concern (Makau et al., 2016). AFM1 is the major metabolite of AFB1 in mammals; it is partially excreted into milk, and is a possible human carcinogen. Milk yield and the carry-over of AFB1 in the feed to AFM1 in the milk are highly correlated, so the definition of the AFM1 carry-over in dairy cows is considered of extreme importance.

A population of Israeli-Holstein cows of high milk production (world record milk production) (Britzi et al., 2013) were used to determine AFM1 carry-over following daily oral administration of feed containing ~86 μg AFB1 for 7 days. The carry-over appears to increase exponentially with milk yield and is described by the equation: carry-over% $= 0.5154\, e^{\,0.0521} \times$ milk yield, with $r^2 = 0.6224$.

The monitoring of AFM1 in animal milk is an official control activity performed by the Member States to verify that the provisions set by the EU food low legislation are enforced and complied. The AFM1 limit in cow milk set in EU is a legislative reference also for other countries. In India, Nile (Nile et al., 2015) monitored a total of 600 samples of milk from buffalo, cow, goat, and sheep from urban, semi-urban and rural areas for the content of AFM1 in milk. In this work, it is shown that AFM1 contamination was found in 36% of the samples of which 16% of buffalo, 44% of cow, 10% of goat, and 12% of sheep milk samples were above the maximum limit accepted by the EU. Both in the Nile et al. monitoring study and the in the Kenyan study (Makau et al., 2016), the level of AFM1 concentration was found minimal in milk coming from rural areas.

Oral supplementation of clay (at 0.5%, 1%, 2%) to dairy cattle has been reported to reduce toxicity of AFs in contaminated feed (100 μg of AFB1/kg of dietary dry matter intake) (Sulzberger et al., 2017). Concentrations of AFM1 in milk, AFB1 in faeces and AFB1 in rumen fluid were reduced in cows fed with clay compared with positive control with no clay.

Multi-mycotoxins

The consumption of feeds contaminated with mycotoxins (particularly AFs, OTA, and ZEN) by the animals causes various hepatic and renal disorders, severe immunodeficiency and reproductive disorders. The presence of these mycotoxins in different organs depends on the selective place of metabolisation of these mycotoxins (Simion et al., 2010). Various studies have determined the presence of AFM1 and of ZEN in milk, of AFB1 in the hepatic tissue and in the serum, of OTA in the liver, of ZEN in the bile. The presence of AFs (AFB1, AFB2 and AFM1), OTA and ZEN, or their metabolites, was investigated in various bovidae fluids (milk, ruminal fluid, urine and bile) by Simion (Simion, 2010). The presence of mycotoxins resulted in all the analysed matrices: total AFs and OTA in the ruminal fluid, AFM1 in milk. In contrast AFB1, AFB2 and ZEN were absent in the samples of urine and bile.

High-production dairy and beef farming systems require the supplementation of diets rich in starch. This practice may induce ruminal acidosis and also increase exposure to mycotoxins because starches in starch-rich diets are the main vehicles of mycotoxin contamination (Pantaya et al., 2016). The effects of low ruminal pH on the bioavailability of 4 major mycotoxins AFB1, OTA, DON, FB1 was scrutinized by Pantaya (Pantaya et al., 2016) in a feeding trial of high and low starch diet with and without yeast supplementation contaminated by AFB1, OTA, DON, FB1. In conclusion, after fecal and urinary analyses it came out that the bioavailability of DON and FB1 remained unchanged; in contrast, high-starch diets increased the OTA and AFB1 bioavailability, most probably through the lowering effect on ruminal pH. Urinary excretion of OTA after mycotoxin administration increased in the high-starch diet, correlated with lower fecal excretion. Similarly, a decrease in

fecal excretion of AFB1 was accompanied by an increase in urinary excretion of its major metabolite, aflatoxin M1.

In a study (Hashimoto et al., 2016) where cows were supplemented with OTA contaminated diet (5–100 µg/kg), OTA appears not to be carried over into milk and tissues (such as liver, kidney, muscles, fat and jejunum ileum) of cows. In contrast, a small amount of OTA (0.1 µg/kg) was detected in the blood plasma concluding that the ingestion of diets containing up to 100 µg/kg of OTA over 28 days does not affect feed intake or milk production of cows and the dietary OTA is not carried over into milk and edible tissues such as liver, muscles and fat.

The increased plasma concentrations of ZEN, DON and DOM1 may hint on toxin exposure through the diets. Urinary ZEN levels decreased when the rice straw feeding contaminated with ZEN was substituted with new rice straw with lower ZEN contamination (Hasunuma et al., 2012). A linear relationship between toxin intake and plasma levels was established by Winkler (Winkler, 2014). DON, DOM1 and ZEN levels were measured in plasma of dairy cows supplied with diets DON and ZEN contaminated close to the current guidance values and the authors (Winkler et al., 2014) established that DON, DOM1 and ZEN were detected simultaneously in all plasma samples but the average performance level (e.g. daily dry matter intake, energy balance and milk yield) was not affected by DON and ZEN levels in feed.

3.6.2.4. Rats and mice

Mice and rat were included in this group that presented only toxicokinetic study with data on BM. As it is shown in Figure 21, among all the publications considered DON is the higher mycotoxin represented while faeces and urine are the most common analysed substrates.
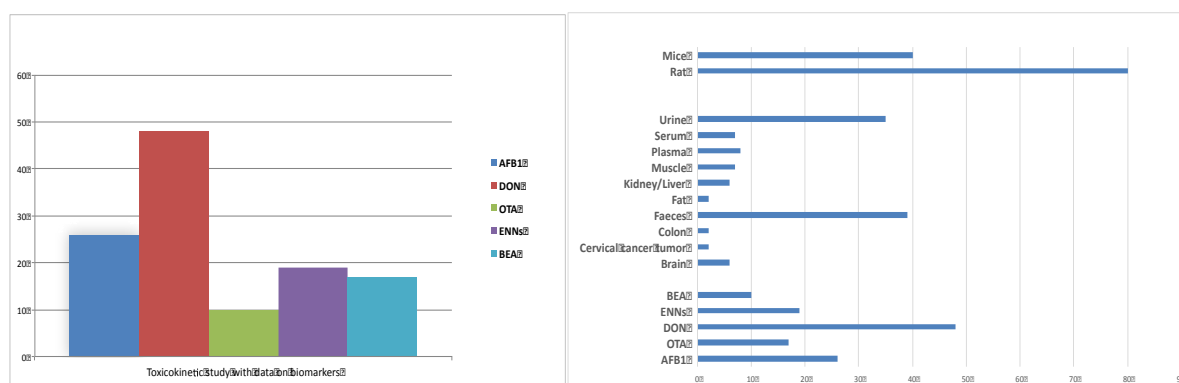
Figure 21: Distribution of biomonitoring and toxicokinetic studies in rat and mice reporting markers of exposure and the number of BM measurements for rat and mice species, organs and mycotoxin congeners

Aflatoxins

Huang (Huang et al., 2017) investigated the potential binding capacity of *Lactobacillus plantarum* isolated from Chinese traditional fermented foods to reduce the toxicity of AFB1 toxicity, and its subsequent detoxification mechanism. The *L. plantarum* strains were orally administered to mice with liver oxidative damage induced by AFB1. *L. plantarum* may alleviate AFB1 toxicity by increasing fecal AFB1 excretion, reversing deficits in antioxidant defense systems and regulating the metabolism of AFB1.

Brain concentration of AFB1 was used to evaluate the modulation of AFB1 passage into the brain. Tras (Tras et al., 2017) conducted the first *in vivo* experimental study intended to verify if plasma and brain concentrations of AFB1 are affected by the modulation of trans-membrane proteins, namely the ATP-binding cassette transporter superfamily (e.g. P-glycoprotein and the breast cancer resistance protein), by using 2 drugs:

prazosin, an alpha-adrenergic blocking agent, and Zosuquidar, a compound of antineoplastic drug candidates. Both drugs significantly reduced the brain concentration of AFB1 but not the plasma concentration. They evidenced that AFB1 may be a substrate of both P-glycoprotein and the breast cancer resistance protein (P-gp and BCRP).

Rat blood and faeces were analysed to verify the efficacy of the oxidized tea polyphenols (OTP) to complex AFB1. Lu et al. (2017) demonstrated that AFB1 can be complexed and the absorption of the complexed-AFB1 is inhibited in rats. In fact, AFB1 plus OTP group had significantly ($P < 0.05$) decreased AFB1-albumin compared to the AFB1 group at 4 h after ingestion significantly promoting ($P < 0.01$) the elimination of AFB1 in faeces (Lu et al., 2017).

Enniatins and beauvericin

The *Fusarium* toxins ENNB and BEA have recently aroused interest as food contaminants but data about their toxic profile are limited. Short and long exposure were studied for these two *Fusarium* toxins.

Both *Fusarium* toxins were investigated by Rodríguez-Carrasco (Rodriguez-Carrasco et al., 2016) in mice treated intraperitoneally. ENNs and BEA were found in all tissues and serum but not in urine. Moreover, they were measured in liver and fat demonstrating the molecules' tendency to bioaccumulate in lipophilic tissues. While for BEA no metabolites could be detected, for ENNB phase I metabolites were found in liver and colon, namely deoxygenated-ENNB, mono- and di-demethylated-ENNB. As it is reported in table S11 the deoxygenated-ENNB gave the higher quantitation indicating that the contribution of hepatic and intestinal metabolism seems to be involved in the overall metabolism of ENNB.

In a study performed by Juan (Juan et al., 2014), Wistar rats were dietary supplemented with ENNA during 28 days of exposure time to determinate its levels in serum, urine and faeces and to evaluate the immunologic effect in peripheral blood lymphocytes (PBL).

Ochratoxin A

OTA and OTα in urine and faeces were measured in Fisher rats (Abbas et al., 2013), which were allocated with dietary treatments consisting of flavonoid-free balanced diet containing 10 mg OTA/kg and the same diet supplemented with 100 mg quercetin/kg. OTA was mainly excreted as OTα (93%), and only a small part (6–7%) appeared in its original form. Similar as in urine, OTα and OTA accounted for 94% and 6%, respectively, in faeces. Quercetin supplementation had no effect ($P > 0.05$) on feed consumption, OTA-intake, water intake and body weight gain. Faecal and urinary excretion of OTA and OTα and concentrations of OTA in all tissues were not affected by quercetin supplementation concluding that the polyphenol quercetin has no impact on the toxicokinetics of OTA in vivo.

Deoxynivalenol

DON, DON3G and DON metabolites (deoxynivalenol-glucuronide and DOM1) were monitored in rats' urine and faeces (Nagl, 2012) after administration of DON3G and DON contaminated diet. DON3G was recovered only for the 3.7±0.7% of the given dose in urine in the form of analysed analytes (14.9±5.0% after administration of DON, and only 0.3±0.1% were detected in the form of urinary DON3G). The majority of administered DON3G was recovered as DON and DOM1 in faeces. These results suggest that DON3G is little bioavailable, hydrolysed to DON during digestion, and partially converted to DOM1.

3.6.2.5. Other animal species: horses and dogs

Ochratoxin A and zearalenone

OTA was determined in blood samples of healthy and affected by chronic kidney disease dogs (Meucci et al., 2017). CKD group showed higher incidence of OTA than healthy dogs (96 vs. 56%) and a significantly higher median value of OTA plasma concentration (0.008 vs. 0.144 ng/ml). While plasma, urine and faeces were assessed to define the ZEN and metabolite contents in horses fed with contaminated oats (Songsermsakul et

al., 2013). The study concluded that the main conversion of ZEN was into βZEL in horses explaining this animal species is not susceptible to ZEN in comparison with swine. The βZEL levels found in plasma were detected at higher level on day 10 of the study. βZEL and αZEL were the major metabolites in urine and ZEN, βZEL and αZEL were predominantly found in faeces. The degree of glucuronidation was established in all sample types, approximately 100% in urine and plasma, whilst low glucuronidation was found in faeces samples.

3.6.3. Biomarkers of exposure in humans

The focus of the reviewed literature was addressed for selecting all studies where the biomarker was used for assessing human exposure and for driving knowledge of all the possible correlations between mycotoxin exposure rates of intake and health effects. The selection criteria followed included the kind of bio-monitoring studies that were planned to study either the parent molecule or its metabolites, especially in multi-toxin measurements. A number of studies that were published for the purpose of the method setting/validation, was also taken into consideration. This is considered a topic of crucial importance in the bio-monitoring field, in fact, whenever available, performances of the method (LODs and LOQs and validation performances) were also included to substantiate the reliability of the method.

The final number of the references about the human studies after the selection criteria summed up 176 articles focused on biomarker studies of a single mycotoxin or of multi-mycotoxin. The 176 articles produced more than 2500 records. It was defined as a multi-biomarker study that one where the parent and one or more metabolite was analysed; thus, the number of multi-biomarker measurements accounted for 37.5%, the remaining 62.5% regarding publications that reported studies of a single mycotoxin.

Table 16 summarizes the number of records registered for each mycotoxin, the number of references and the number of combinations where the toxin was studied in a single or multi analysis (either in combination with its metabolites only or with other mycotoxins). As it is shown the number of publications on aflatoxins are the majority followed by DON, OTA, FBs and ZEN. There are a number of other mycotoxins (i.e. ALT, TEA, FUSX, NEO, CIT, NIV, T2, DAS, ENNs) that have been scrutinized in quite a few publications.

Figure 22 synthesizes the number of the total references that populates the human dataset indicating the distribution between the two groups of studies, i.e. single and multi BM study. The figure shows also the distribution of number of references reporting single BM, a multi BM with the parent plus its metabolites, and multi-mycotoxin.

Table 16: Summary of mycotoxins, number of records, number of references and the type of study present in the dataset of studies on biomarkers in humans

Mycotoxin	N° records	N° of references (a)	N° of references with a single-BM	N° of references with a multi-BM (b)
AFs	453	102	74	12/16
ALT	2	1	-	-/1
OTA	199	45	23	4/18
DON	279	31	3	11/17
FBs	100	22	5	3/14
TEA	5	2	1	1/-
FUSX	7	2	-	-/2
NEO	4	2	-	-/2
CIT	51	9	-	3/6
NIV	10	4	-	-/4
T2	17	4	-	-/4
ZEN	142	19	-	5/13
DAS	7	2	-	-/2
ENNs	4	1	-	-/1
Multi-BM	805	66		
Single-BM	454	110		

a) Number of references where the parent mycotoxin or a metabolite has been considered either in a single or multi-/BM study
b) Parent plus its metabolites/multi-mycotoxin
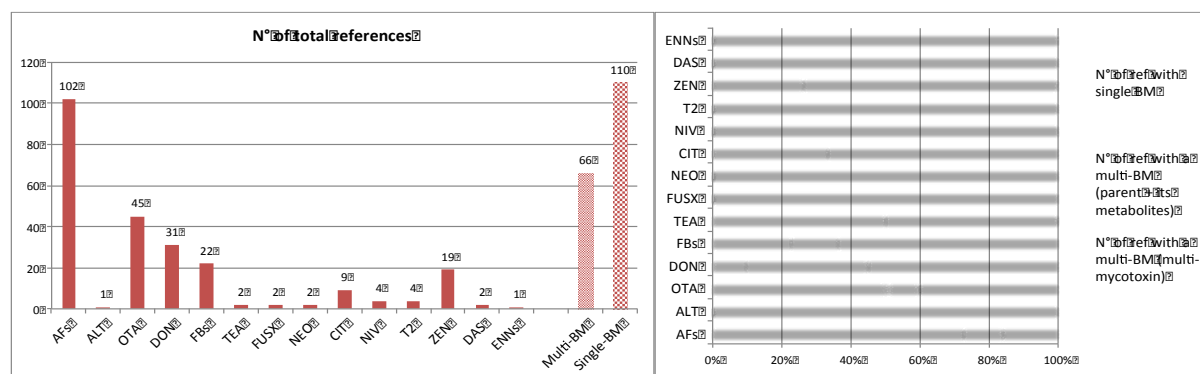
Figure 22: a) The number of the total references in the human dataset and the distribution between the single biomarker study and multi biomarker study (left) and the distribution of number of references for each mycotoxin reporting single biomarker data, multi biomarker data, i.e. parent + its metabolites, and multi-mycotoxin data (right).

Supplementing tables (L1-L6) are reported. Supplementing tables summarize the single biomarker history for AFs (L1), OTA (L2), DON (L3), FBs (L4), ZEN (L5), CIT and TeA (L6). Supplementing tables report the substrate, the population group, the analytical method, the range of values, the information on the study, the association with the diet and the exposure values (when available) for each BM in the reference. Moreover, supplementing table L7 collates the same BM info extracted specifically from multi-biomarker studies.

3.6.3.1. Aflatoxins

In the dataset, the largest number of publications examines AFs. This is understandable due to the toxicological concern of AFB1. The aflatoxin alb adducts in serum (more specifically the aflatoxin-lysine adduct) and

aflatoxin B1-N7-guanine, aflatoxins-mercapturic acid and aflatoxin M1 in urine (Zhu et al., 1987; Gan et al., 1988) represent the first BM of exposure that were validated and used for the mycotoxin risk assessment. The majority of the data in the dataset regards AFM1 analysed in urine (66%), in breast milk (28,5%) and in serum (5,5%). In fact, due to the long half-life of alb in humans, the measurement of aflatoxin alb adducts in blood and its derivatives is strongly preferred with respect to urine, and it indicates an exposure extent over a period of 1-2 months (Sabbioni et al., 1987; Leong et al., 2012). However, AFB1, AFB2, AFG1, AFG2, AFM2, AFalb, AFB1lysine, AFB1guanine and AFOH are also extensively considered. Figure 23 shows the number of measurements of AFs metabolites in the dataset. The substrates analysed are also reported.
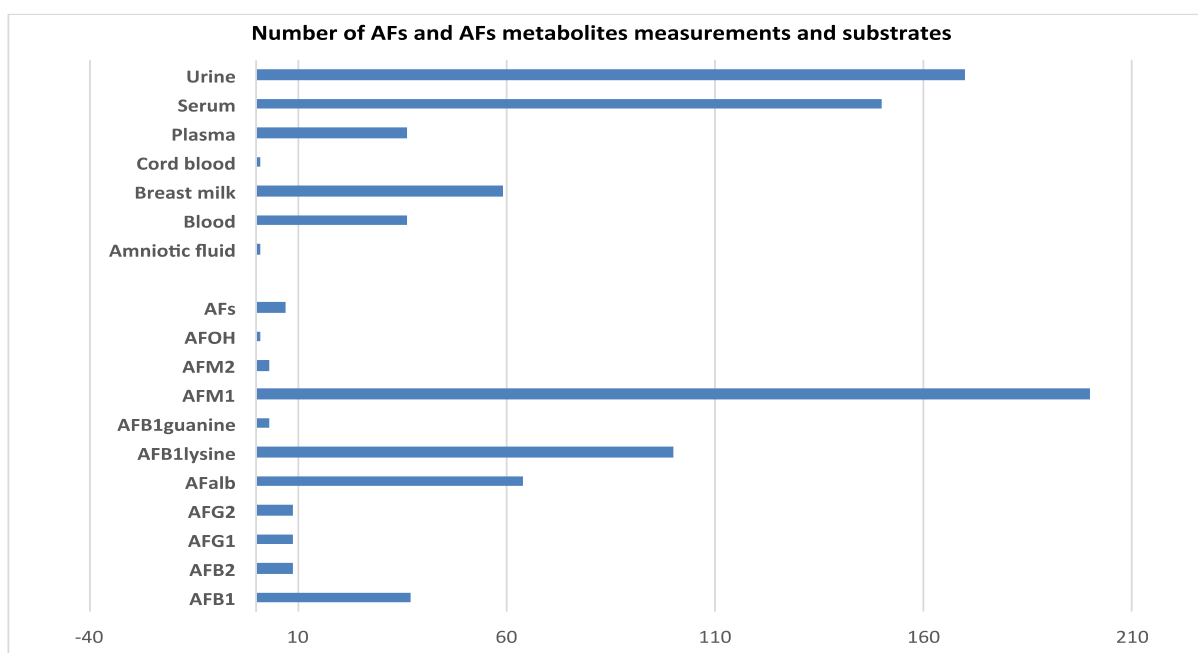
Figure 23: Number of measurements of AFs metabolites and substrates analysed

As regards the BM analytical technique, 41% of the works used ELISA methodology, 32% HPLC-FLD, 21% LC-MS or LC-MS/MS, and 6% used alternative techniques (RIA, TLC, AflaCheck test or VFM). In 28% of publications, lamentably, neither LOD nor LOQ of the method were reported.

In Annex L, supplementing table L1 reports the substrate, the population group, the analytical method, range of values, the information on the study, the association with the diet and the exposure values (when available) for each single biomarker in the reference.

3.6.3.2. Ochratoxin A

Given the extensive occurrence of OTA, which is found in a wide range of foods (i.e. cereals, dried vine fruit, wine, coffee, and liquorice), molecular epidemiology studies are particularly important and helpful for biomonitoring the extent of the exposure.

OTA is present in 45 publications of which 23 are about single mycotoxin exposure study, 4 are about OTA and metabolites and 18 are publications where OTA is analysed in a multi-mycotoxin study. As for AFs, OTA has been studied in a wide range of substrates (see Figure 24 and table L2 in ANNEX L). OTα, the hydrolysed form of OTA, is the unique metabolite that is reported in the dataset; however, OTαAglicone and 2R'OTA are also measured. In addition to urine and serum, OTA levels have been measured also in breast milk, especially to assess the exposure of a particular subgroup (lactating women) and to assess the potential exposure of the

offspring. Up to now, the pattern of OTA distribution in milk is still unclear and few works attempted to correlate the dietary intake with OTA concentration in milk, nevertheless it is to highlight that, being the new-borns exclusively breast fed during the first weeks, the biomonitoring of OTA in human milk is an assessment of the risk for infants (Valitutti et al., 2018).

OTA analyses are mainly carried out by HPLC-FLD (61%). When multi mycotoxin are analysed, LC-MS/MS is the preferred analytical technique. However, still a number of ELISA methods are carried out (29%). In 9 of the publications there is a lack of information about LOD or LOQ.

Table L2 shows the list of substrates and the range of levels found for OTA and OTA metabolites, table L7 shows the synthesis of the papers where OTA was studied in a multi-mycotoxin work.

The Klassen and Breitholtz equation (Gilbert et al., 2001; Scott, 2005) is currently used to obtain the estimate exposure values to OTA starting from plasma concentrations (ng/ml from serum analyses), plasma clearance (i.e. renal filtration rate) and OTA bioavailability (considered for most animals of 50%). It is noteworthy to mention that recent comparative studies failed in correlating blood levels and dietary intake (Duarte et al., 2010) probably due to the lack of information on the toxicokinetic mechanisms and the fact that the long half-life and continuous OTA exposure result in a steady state concentration (EFSA, 2006). Hence, the use of OTA in blood as validated biomarker is substituted by the measurement of urinary OTA whose content is lower but constitutes a promising alternative especially when further developments on the relationship between OTA intake and the urinary biomarker will be defined.
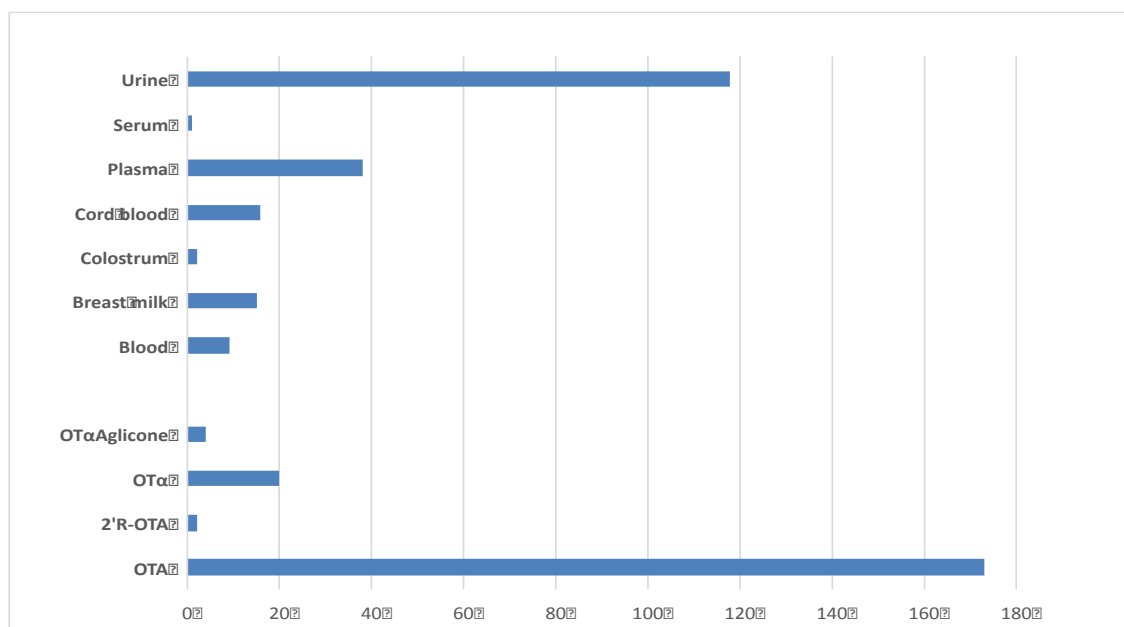
Figure 24: Number of measurements of OTA and OTA metabolites and substrates analysed

3.6.3.3. Deoxynivalenol

DON is present in 31 publications of which 3 are single mycotoxin exposure study, 11 are about DON and metabolites and 17 are publications where DON is analysed in a multi-mycotoxin study. In the dataset considered, DON has been studied only in urine and in fact, it was estimated that 70% of the ingested DON is excreted to urine. DON is partially de-epoxidised into DOM1 metabolite and the main biotransformation of DON is conjugation with glucuronic acid leading to the presence of DON3glucur, DON15glucur and also DOM1glucuronides were analysed (Turner et al., 2010; Turner et al., 2011; Brera et al., 2015; Ali et al., 2016).

Due to the difficulty to analytically distinguish the two glucuronide forms, often publications report methods which imply the use of a double extraction procedure with and without enzymatic treatment for de-

glucuronization; this leads to obtain a total DON measurement from which by subtraction of the free DON contribution, the glucuronidized form contribution is obtained (Turner et al., 2011; Cunha and Fernandes, 2012; Brera et al., 2015).

Warth (Warth et al., 2016) reported for the first time a 3-sulfate form of DON in human urine; 70% of the urine samples of 40 individuals' cohort study, resulted positive to DON3Sulfate while none of the samples was found positive to DON15S. The exposure to DON acetylated forms was also explored in human urinary samples: Rodriguez-Carrasco (Rodriguez-Carrasco et al., 2014) found any positivity while Duringer et al. (2016) found one positive sample to 3AcDON (Duringer et al., 2016).

Excepting for 3 works (10%), which used GC chromatography coupled with spectrometric detection, DON and its metabolites are analysed by LC-MS/MS (90% of the publications) and all reported LOQ/LOD values.

In ANNEX L, table L3 shows the list of substrates and the range of levels found for DON and DON metabolites, table L7 shows the synthesis of the papers where DON was studied in a multi-mycotoxin work.

Deoxynivalenol contamination is associated to wheat and maize crop, thus the exposure is commonly linked to cereal based diets. In this regard, a dietary intervention study demonstrated that avoiding wheat reduced significantly the urinary levels of DON (Turner et al., 2008). DON, DON glucuronide and DOM1 may be present in urines of exposed individuals, and as shown in the table L3 a number of studies have been put in place to monitor the BM especially in those areas whose climatic conditions favour the proliferation of *Fusarium* species responsible of DON production.
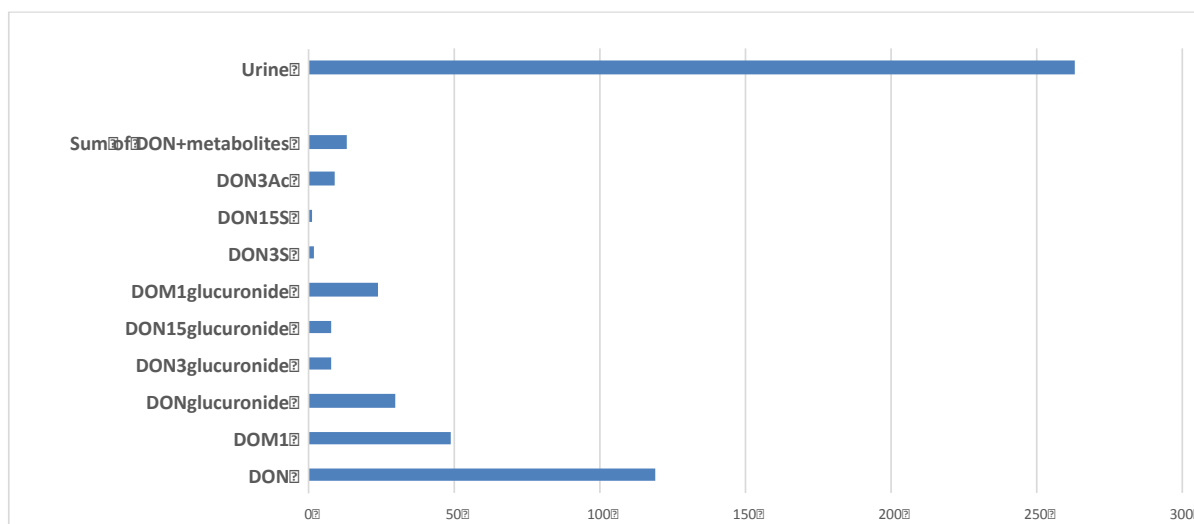
Figure 25: Number of measurements of DON and DON metabolites and substrates analysed

3.6.3.4. Fumonisins

FBs are present in 22 publications of which 5 are single FB1 exposure study, 3 are about FB1, FB2, FB3, Sa, So, while 14 are publications where FB1 is analysed in a multi-mycotoxin context study. In the dataset considered, FBs have been studied in urine mainly, one study detected FB1 in faeces (Phoku et al., 2012) and one in breast milk (Magoha et al., 2014).

The research on fumonisins has been sought for long time in order to define putative BM. The metabolism of fumonisins is limited, FBs are poorly absorbed, distributed and eliminated rapidly by the organism. The oral bioavailability is generally below 5% for FB1, and the measurement of the free urinary fumonisins (B1 and B2) seems to be a choice for biomonitoring the exposure with sensitive techniques. Van der Westhuizen (van der Westhuizen et al., 2011) presented a study where the relationship between urinary FB1 and FB1 ingestion gave good results, confirming the potential use of free FB1 as a validated biomarker.

Sphinganine (Sa) to sphingosine (So) ratio has been alternatively proposed as putative biomarker on the basis that FB1 potently inhibits the enzyme ceramide synthase which catalyses the acylation of Sa and re-acylation of So. This inhibition accumulates intracellular Sa altering the Sa/So ratio. In a large range of animal species, the changed ratio occurs in a dose dependent manner, but the same ratio in humans is a debate (Shephard et al., 2007). Plasma has been explored for the Sa and So levels under this hypothesis (van der Westhuizen et al., 2010; Cano-Sancho et al., 2011).

Excepting for one article, which used ELISA, the 99% of the studies carried out the analyses with HPLC-FLD (53%) and LC-MS/MS (46%). Almost all the articles (excepting 4) reported LOQ/LOD values.

In ANNEX L, table L4 shows the list of substrates and the range of levels found for fumonisins as biomarker.
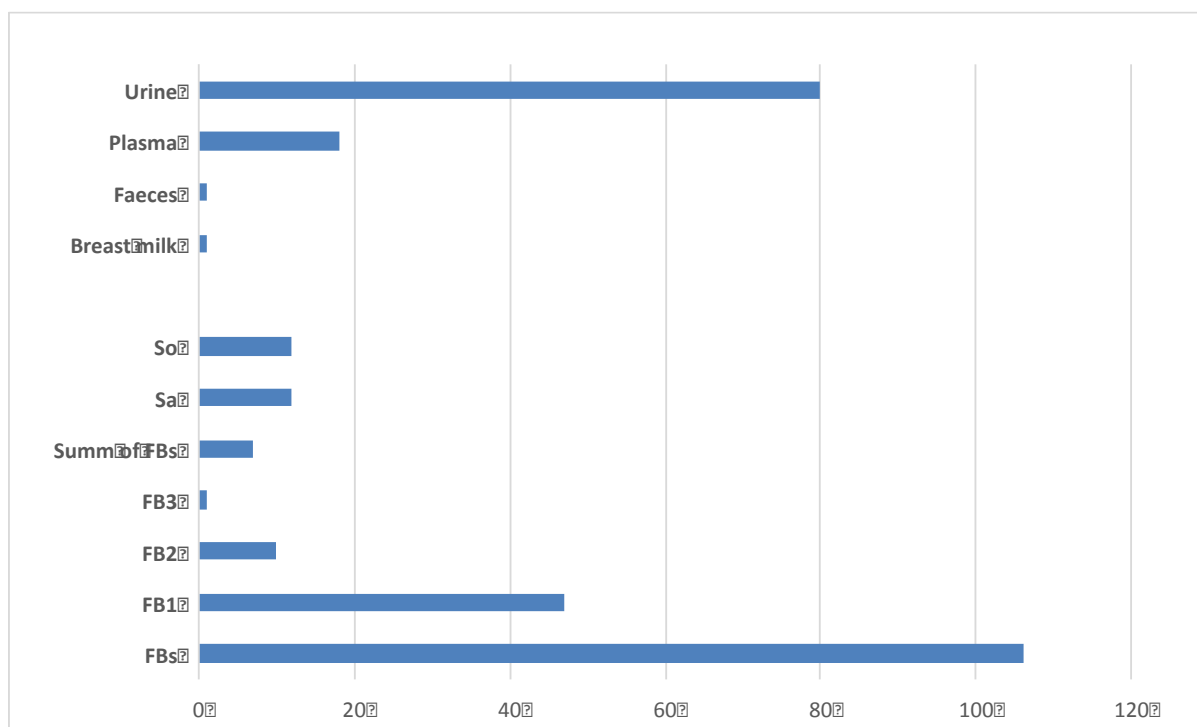
Figure 26: Number of measurements of FB1, FB2, FB3, Sa and So and substrates analysed

3.6.3.5. Zearalenone

ZEN is present in the dataset with a high number of metabolites. Figure 27 shows the kind of substrates for ZEN and the list of ZEN metabolites measurements in the dataset. In total 19 studies were selected for ZEN, and its metabolites. The majority of the studies take into consideration ZEN and its phase I metabolites (ZAN, α- and β- zearalenols and zearalanols). There is only one study in the dataset who analysed ZEN together with its phase I and glucuronides forms (Mauro et al., 2015). In a quite high number of publications (74%), ZEN was included in a multi-mycotoxin study (with 2 to 6 other mycotoxins) where phase I metabolites were always considered, often including glucuronides. ZEN, ZAN, α- and β-ZEL, α- and β-ZAL, ZEN4G, ZEN14Oglucuronides have been always quantified in urine (100%), in 2 cases also in serum (Mauro et al., 2015; Fleck et al., 2016). The glucuronide forms ZEN14 ZEN14Oglucuronide was analysed by four authors (Abia et al., 2013; Ezekiel et al., 2014; Gerding et al., 2015; Cirlini et al., 2017).

The 100% of the studies carried out the analyses by LC-MS/MS but one fourth of the articles did not report information about LOQ/LOD values.
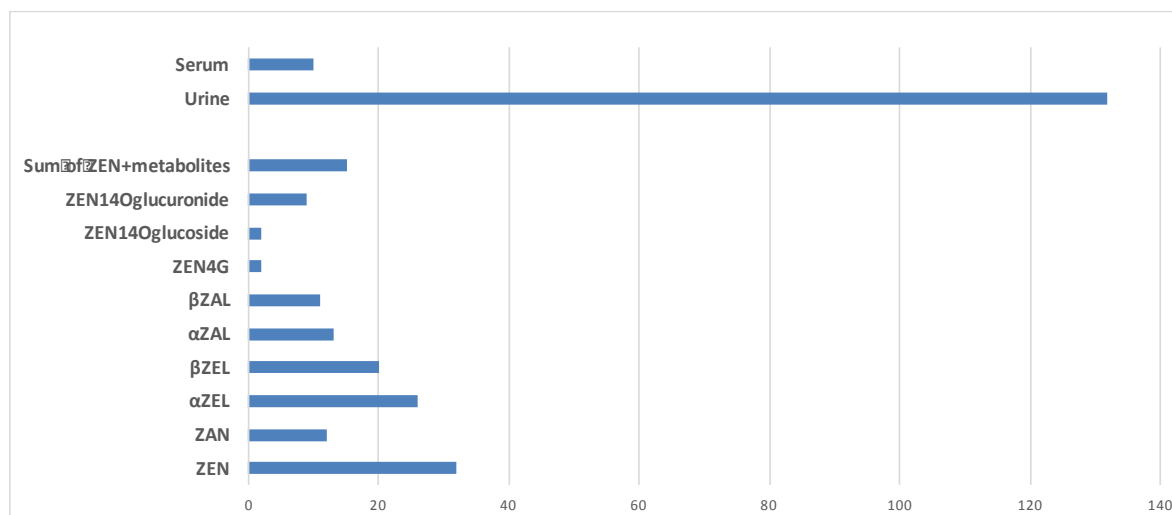
Figure 27: Kind of substrates for ZEA and number of ZEN and ZEN metabolites measurements in the dataset

Table L5 shows the list of substrates and the range of levels found for ZEN as biomarker.

3.6.3.6. Citrinin and tenuazonic acid

Citrinin has been only recently introduced as biomarker in exposure studies. In the available dataset, there are 9 works of which 3 papers investigated CIT and CIT metabolite, dihydrocitrinone (OHCIT), while 6 publications have introduced CIT in a multi-mycotoxin study. CIT is excreted in urine as such and as OHCIT in above 70% of the subject studied (Blaszkewicz et al., 2013; Ali et al., 2015a; Ali et al., 2015b), either adults and children (Blaszkewicz et al., 2013). The studies were carried out using HPLC-FLD and LC-MS/MS, one study did not report LOD/LOQ values.

Tenuazonic acid (TeA) is one of the *Alternaria spp*. mycotoxins that may be present in cereals as well as in tomatoes and respective processed products. The two publications reporting urinary TeA showed that it was detectable in all German subjects. Moreover, Hövelmann (Hövelmann et al., 2016) showed a trend regarding elevated intake of cereal products and higher excretion of tenuazonic acid. The epimerization product of TeA under basic or acidic conditions forms alloTeA, thus seems to be of upmost importance to measure the presence of both isomers in risk assessment context.

Table L6 summarises the publication information about CIT and OHCIT and TeA.

3.6.3.7. Multi-mycotoxins

The multi-biomarker measurement was realized in 66 of the publications reported. The parent molecule and its metabolites or groups of 2 up to 7 mycotoxins have been analysed in biological fluids as shown in Figure 28. Of the 27 studies reporting multi-mycotoxin, different combinations were registered as it is shown in Table 17.

The simultaneous determination of more than one mycotoxin in human biological fluids by means a multi-biomarker method is a new challenge in mycotoxin biomonitoring. Especially urine seems to be the preferred specimen either for the easiness and approachability of the matrix and for its handy non-invasive collection, better accepted by study participants. Besides, kidneys are the primary routes for the elimination of those xenobiotics that for their chemical characteristics are excreted in urine as parent mycotoxins or conjugated forms. However, serum, plasma and breast milk are also studied substrates in multi-mycotoxin analyses. As it is shown in Figure 28, AFs and DON together with their metabolites are the most studied.

Table L7, in ANNEX L, summarized the combination of the multi mycotoxins studies.

In the last 5 years the number of multi-mycotoxins scientific works have been increasing and improving thanks to the accessibility of reliable techniques and methods of analysis. Thus, if the multi-mycotoxin analyses are very helpful, still there is a lot of work to be done to cope with several constraints either in the analytical context (the lack of method standardization, the unavailability of commercial reference standards, especially glucuronides) or in the exploitation of the biomarker in the exposure assessment. In fact, the lack of information on the human bioavailability of the toxin, the excretion rate, the definition of the right biomarker (i.e. validated biomarker) to be used represent knowledge gaps for the use of biomonitoring in exposure assessment.
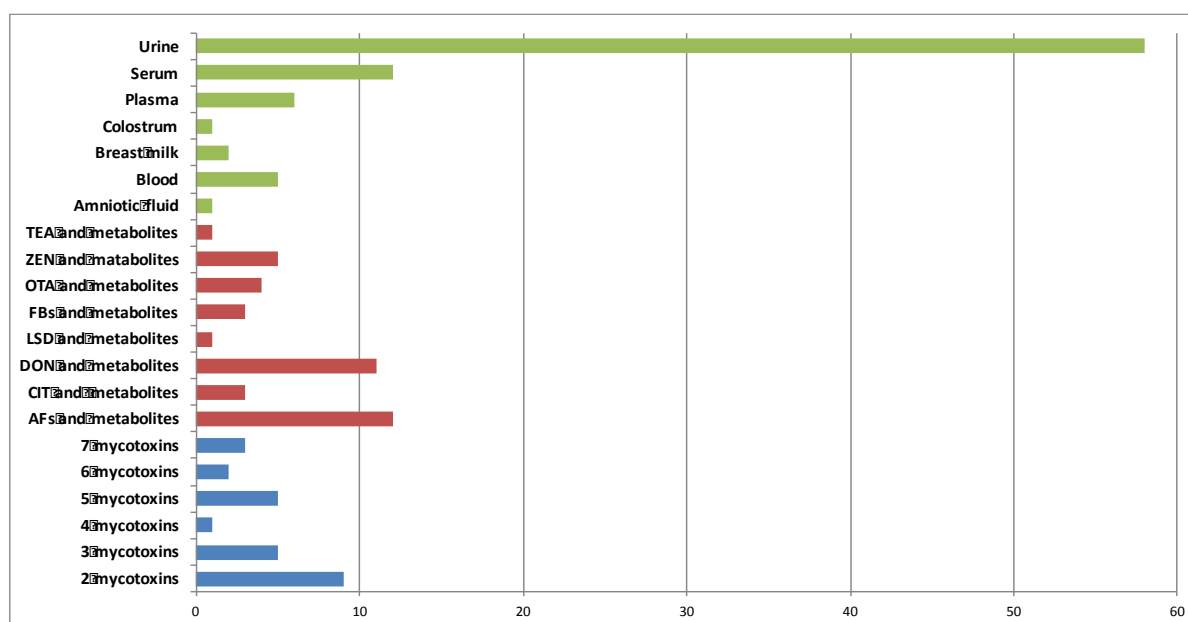
Figure 28: Kind of substrates for ZEA and number of ZEN and ZEN metabolites measurements in the dataset

Table 17: Summary of the combination on the multi-mycotoxin studies

2 mycotoxins	3 mycotoxins	4 mycotoxins	5 mycotoxins	6 mycotoxins	7 mycotoxins
OTA, CIT	AFM1, FBs, OTA	CIT, DON, OTA, ZEN	AFs, DON, FBs, OTA, ZEN	AFs, DON, FBs, NIV, OTA, ZEN	AFs, CIT, DON, ENNs, FBs, OTA, ZEN
AFM1, OTA	DON, FBs, ZEN	-	CIT, DON, ENNs, T2, ZEN	AFs, DON, FBs, OTA, T2, ZEN	DAS, DON, FUSX, NEO, NIV, T2, ZEN
CIT, OTA	AFS, FBs, OTA	-	DON, FBs, NIV, OTA, ZEN	-	DAS, DON, FUSX, NEO, NIV, T2, ZEN
AFs, DON	ALT, DON, ZEN,	-	-	-	-
AFs, FBs	AFM1, OTA, DON	-	-	-	-

3.7. Modelling framework

3.7.1. Model selection and its application to risk assessment of mycotoxin mixtures: a data mining problem

3.7.1.1. Toxicokinetics (*in vivo*) database: internal dose calculation

Regarding the toxicokinetic database *in vitro*, only 3 studies report on F (bioavailability), while within data extracted and available in toxicokinetic database *in vivo*, only the following papers report data for all the parameters necessary for calculating the internal dose (F-bioavailability, Ke-rate of elimination, CL-clearance, Vd-volume of distribution, Tmax-maximum concentration, Kabs-rate of absorption).

a) Swine DON, (Saint-Cyr et al., 2015)
b) Swine DON, (Paulick et al., 2015)
c) Chicken DON, (Osselaere et al., 2013)
d) Chicken ZEN, (Buranatragool et al., 2015)
e) Chicken T2, (Sun et al., 2015)
f) Chicken NIV, (Kongkapan et al., 2015)
g) Chicken ENNB ENNB1 (Fraeyman et al., 2016)
- PIGS + DON

All the chemical properties of mycotoxins for internal dose modelling were recovered and are available online on the MYCHIF repository and EFSA knowledge junction (10.5281/zenodo.3615174).

For this attempt, we implemented both Toxicokinetic model (1-cmpt approach) and Toxicodynamic model (multi-cmpt approach). For toxicokinetic model and DON-swine case study we have also performed a global sensitivity analysis (GSA, FAST99) (Saltelli et al., 1999). All the r-code and model outputs are available are available online on the MYCHIF repository and EFSA knowledge junction (10.5281/zenodo.3615174). Unfortunately data availability in the literature and the great heterogeneity of the reported case studies did not allow to proceed further with these modelling tool.

TK 1cmpt Swine DON po Saint Cyr et al 2015 doi:10.3390/toxins7124873
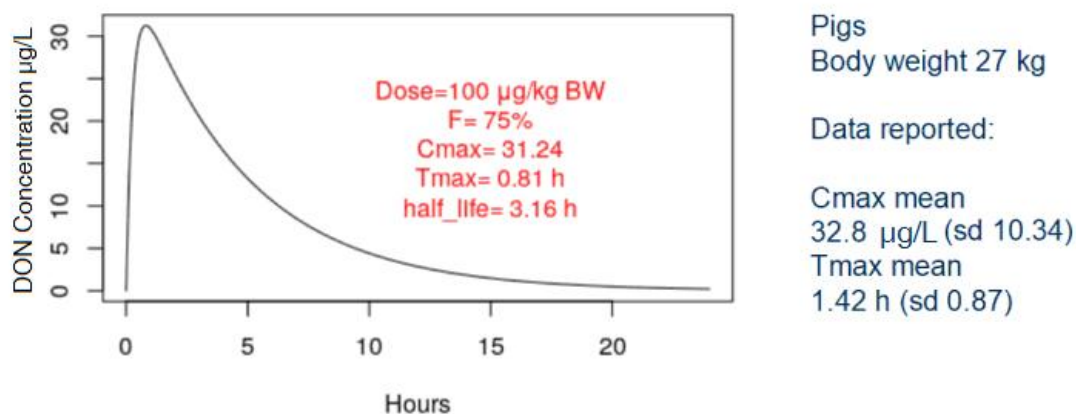
Figure 29: Toxicokinetic model 1-cmpt, output and comparison with data reported by (Saint-Cyr et al., 2015) DON-swine oral administration.

Data reported by (Saint-Cyr et al., 2015) are used for model initialization.

Chicken ZEN Buranatragool et al 2015 doi:10.1016/j.toxrep.2014.12.011
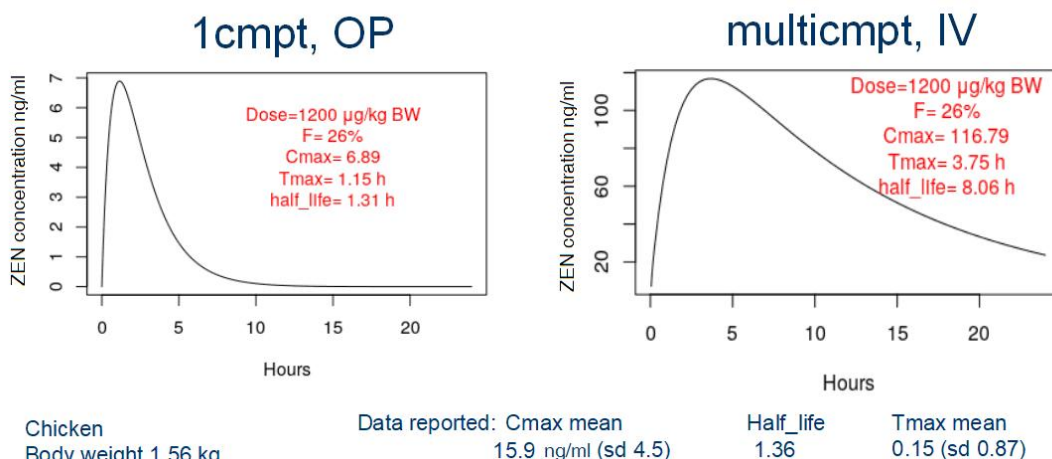
Figure 30: Toxicokinetic (1-cmpt) and toxicodynamic (multi-cmpt) models output and comparison with data reported by literature. ZEN-chicken intravenous and oral administration.

Data reported by (Saint-Cyr et al., 2015) are used for model initialization.
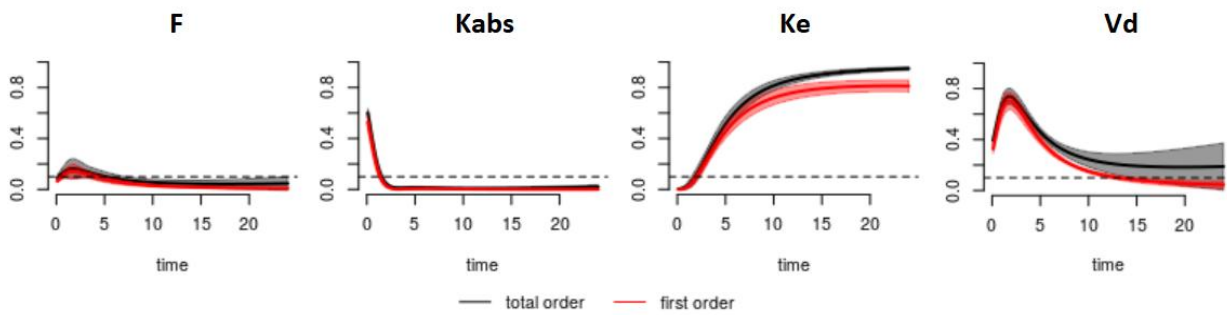
Figure 31: Sensitivity analysis for model input (F = bioavailability, Kabs = absorption rate, Ke= elimination rate, Vd = volume of distribution)- case study SWINE-DON, (Saint-Cyr et al., 2015).

Ke and VD are the most sensitive parameters in this model.

3.8. Case studies

3.8.1. Animal case study

3.8.1.1. Dose-response modelling for mycotoxin mixtures using a component-based approach in pigs and chicken

From the *in vivo* toxicity database, dose response data were modelled for mycotoxin mixtures in chicken and pigs using component-based approaches assuming either dose addition or response addition using the drc R-package (analysis of dose response)(EFSA, 2019). For chicken, 14 combinations of mycotoxins mixtures were extracted and modelled of which one with 3 components and 13 as binary mixtures (Table 18). For pigs, 9 combinations were extracted and modelled of which one with 3 mycotoxins and 5 as binary mixtures (

Table 19).

Table 18: Modelling of multiple mycotoxins in chicken using component-based approach

ID_case	MIXTURE	Target
1	DON,FB,AF	Body weight
2	DON,FB	Intestinal villus height
3	DON,AF	Blood heterophils to lymphocytes ratio
4	AF,FB	Blood alanine aminotransferase
5	AF,FB	Blood albumin
6	AF,FB	Blood aspartate
7	AF,FB	Blood calcium
8	AF,FB	Blood colesterol
9	AF,FB	Blood total protein
10	AF,FB	Blood triglycerides
11	AF,FB	Blood γ-glutamyltransferase
12	AF,FB	Feed conversion ratio
13	AF,FB	Feed intake
14	AF,FB	Liver weight

Table 19: Modelling of multiple mycotoxins in pig using component-based approach

ID_case	Mixture	Target
1	DON,ZEN,AF	Blood aspartate aminotransferase
2	DON,ZEN,AF	Body weight gain
3	DON,ZEN,AF	Feed intake
4	DON,ZEN,AF	Liver weight
5	DON,ZEN	Blood alanine aminotransferase
6	DON,ZEN	Blood alkaline phosphatase

7	DON,ZEN	Blood glutathione peroxidase
8	DON,ZEN	Blood SOD
9	DON,AF	Blood urea nitrogen

Figure 32: Model results for **pig case study1** (DON/ZEN/AF target blood aspartate aminotrasnferase)

All the tables, model parameterisation, plots and R-code are available online on the MYCHIF repository and EFSA knowledge junction (10.5281/zenodo.3615174).

Subsequently, the combination of 2 toxins (OTA and AF) was selected on the basis of the data availability, in terms of dose-response, that enabled a new modelling attempt. It was modelled both the single and the combined toxicity also by the use of Curve Fitting and Mixture Toxicity Assessment - MixTox R-package (Zhu and Chen, 2016). In this case from the toxicity in vivo database the two species with a sufficient amount of data were leghorn chicken and marek chicken.

Data for the two species was not aggregated and they were modelled separately. The following endpoints were considered:

- 5 endpoints per the chicken "marek breed" (bodyweight, feed conversion, feed intake, kidney relative weight, liver relative weight, spleen relative weight)

- 2 endpoints per white leghorn (final bodyweight, feed intake)

Where the standard deviation was available (in one of the two data sets DSs were missing), a data bootstrap (n = 100) was performed for the response data and it was analysed against the doses reported in the database.

Due to the distribution of dose-response data for the combinations of AF & OTA, only the dose-addition modelling framework was implemented.

Marek (feed intake)
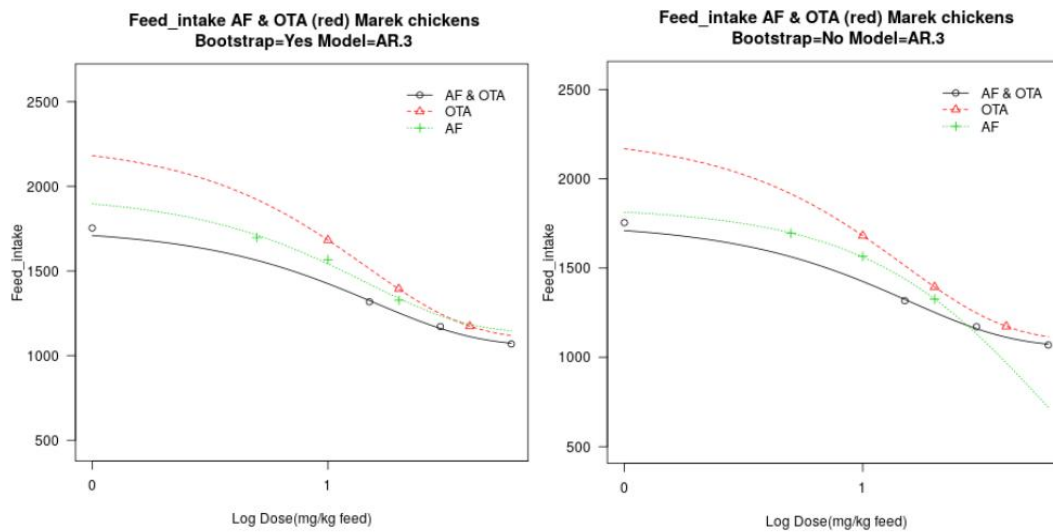
Figure 33: Model results for marek chicken case study: AF-OTA target feed intake

3.8.1.2. Dose-response modelling for mycotoxins in pigs and chicken using benchmark modelling approaches

In the context of mixture toxicology, the combined toxicity of two or more substances may be based on the concept of dose-addition (EFSA, 2008, 2013b). Substances can be seen as dose-additive when they act in a similar manner with the same mechanism/mode of action, but may differ only in their potencies. The concept stipulates that the total effect after simultaneous exposure to such compounds can be estimated from the sum of the doses or concentrations of each component (Zeilmaker et al., 2018). For this purpose, dose-response data were extracted, from *in vivo* toxicity database, for chicken and pig only for three targets Body Weight Gain (BWG), liver and kidney weight and for the available mycotoxins AF, DON, FB, OTA, T2 and ZEN Table 20.

Table 20: Case studies extracted from EFSA Hazards database "Openfoodtox"* for the selected target animal and mycotoxin. Number of studies, number of samples and range of doses were reported.

		BWG			Liver			Kidney		
		N° of study	N of samples	Dose Range [a]	N° of study	N of samples	Dose Range [a]	N° of study	N of samples	Dose Range [a]
Chicken	DON	4	29	4 - 18	3	6	15 - 16	3	6	15 - 16
	AF	13	87	0.05 - 3.5	12	57	0.2 - 3.5	8	51	0.5 - 3.5
	T2	3	18	4 - 6	4	8	4 - 6	4	8	4 - 6
	FB	3	21	50 - 300	6	22	50 - 300	4	11	100 - 300
	OTA	4	24	1 - 4	4	10	1 - 4	4	10	1 - 4
	ZEN	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Pig	DON	3	31	2.5 - 3.5	1	4	3 - 12	1	4	3 - 12
	AF	5	56	0.05 - 3	3	15	0.05 - 2.5	1	9	0.25 - 0.5
	T2	3	36	0.4 - 10	1	5	0.4 - 3.2	1	5	0.4 - 3.2
	FB	4	9	10 - 100	3	10	10 - 100	n/a	n/a	n/a
	OTA	3	9	0.35 - 2	n/a	n/a	n/a	n/a	n/a	n/a
	ZEN	3	17	0.25 - 3	2	7	0.25 - 3	1	4	1 - 3

(a): Excluding controls. Dose unit is mg/kg feed. n/a = data not available

For each dataset, based on different case studies, a mathematical dose-response function was fitted to the data, using fitting models in concordance with EFSA guidelines (EFSA, 2017d) for continuous data. Subsequently, fitted dose-responses were used to calculate the benchmark doses (BMD) for each of the three targets. The BMD is the dose which results in a pre-set effect size or response, the benchmark response (BMR). In this attempt, a BMR5% and BMR10% were applied by using DRC R-package (Ritz et al., 2015) and a BMR based on a biological relevant effect was used (PROAST v65.5) (Table 21).

Table 21: Benchmark dose modelling outputs

	Target	Mycotoxin	BMD5	BMDL5	BMDU5	BMD10	BMDL10	BMDU10	BMDL Proast	BMDU Proast	Variance
Pig											
	BWG	AF	0.11	0.01	0.21	0.12	0.03	0.21	0.70	1.53	0.051
	BWG	DON	2.67	1.07	4.28	2.72	2.56	2.89	n/o	n/o	0.052
	BWG	FB	19.26	8.23	30.29	22.38	9.91	34.85	3.37	9486.00	0.151
	BWG	OTA	0.00	0.00	0.00	0.01	0.00	0.01	n/o	n/o	0.096
	BWG	T2	4.28	1.67	6.88	3.86	2.68	5.04	2.30	4.79	0.041
	BWG	ZEN	0.19	0.08	0.30	0.07	0.03	0.10	0.02	0.69	0.003
	Kidney	AF	0.10	0.07	0.13	0.11	0.07	0.14	n/o	n/o	0.026
	Kidney	T2	3.57	1.83	5.31	2.85	1.79	3.91	n/o	n/o	0.001
	Kidney	DON	n/o	n/o	n/o	n/o	n/o	n/o	n/o	n/o	n/o
	Kidney	ZEN	n/o	n/o	n/o	n/o	n/o	n/o	n/o	n/o	n/o
	Liver	AF	0.10	0.07	0.13	0.11	0.07	0.14	n/o	n/o	0.003
	Liver	DON	n/o	n/o	n/o	n/o	n/o	n/o	n/o	n/o	n/o
	Liver	FB	23.57	21.10	26.05	25.11	22.47	27.76	0.20	74.00	0.032
	Liver	T2	3.57	1.83	5.31	2.85	1.79	3.91	n/o	n/o	0.003
	Liver	ZEN	1.32	0.78	1.86	0.93	0.72	1.14	0.57	1.86	0.001
Chicken											
	BWG	DON	15.06	14.58	15.55	n/o	n/o	n/o	0.02	359.80	0.178
	BWG	AF	0.17	0.17	0.18	n/o	n/o	n/o	0.01	0.98	4.190
	BWG	T2	2.11	1.85	2.37	n/o	n/o	n/o	2.25	4.18	0.350
	BWG	FB	30.55	21.77	39.33	569832	-117457	1257120	0.39	4485.00	0.019
	BWG	OTA	0.00	0.00	0.01	n/o	n/o	n/o	1.30E-05	62.30	0.173
	Liver	DON	15.94	15.27	16.61	15.93	15.27	16.60	n/o	n/o	0.233
	Liver	AF	0.33	0.20	0.46	0.37	0.24	0.51	0.00013	29.33	3.404

Liver	T2	7.06	6.87	7.24	n/o	n/o	n/o	n/o	n/o	0.014
Liver	FB	183.33	136.78	229.88	190.60	139.20	241.99	0.0028	8218.00	0.028
Liver	OTA	1.51	0.82	2.19	1.47	0.86	2.09	0.01	1.73	0.098
Kidney	DON	15.77	15.53	16.01	15.75	15.52	15.99	n/o	n/o	0.187
Kidney	AF	1.89	1.38	2.39	2.53	2.04	3.02	0.00	0.34	0.111
Kidney	T2	4.39	4.23	4.55	4.41	4.25	4.57	n/o	n/o	0.098
Kidney	FB	270.06	261.52	278.59	287.53	279.53	295.52	n/o	n/o	0.380
Kidney	OTA	0.70	0.40	1.00	0.60	0.24	0.97	6.58E-06	59.66	2.233

n/o = no model output

Furthermore the same analysis was performed, but at single case study level, and only for the case studies reporting at least 4 doses (one control + 3 doses) (Table 22). A BMR based on a biological relevant effect was used including BMDL with and without exposure time as covariate (Table 23).

Table 22: Case studies extracted from Toxicitiy database for the selected target and mycotoxins reporting at least 4 doses (one control + 3 doses). Number of studies, number of sample and range of doses were reported.

		BWG			Liver			Kidney		
		N° of study	N of samples	Dose Range [a]	N° of study	N of samples	Dose Range [a]	N° of study	N of samples	Dose Range [a]
Chicken	DON	1	15	4 - 18	n/a	n/a	n/a	n/a	n/a	n/a
	AF	4	31	0.5 - 3.0	4	24	0.5 - 3.0	2	16	0.5 - 2.0
	OTA	2	12	1 - 4	1	4	1 - 4	1	4	1 - 4
		n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Pig	DON	n/a	n/a	n/a	1	4	3 - 12	1	4	3 - 12
		n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

(a): Excluding controls. Dose unit is mg/kg feed. n/a = data not available

Table 23: Benchmark dose model outputs. BMDL_COV and BMDU_COV refer model with exposure time as covariate.

	Target	Mycotoxin	Case study	BMDL	BMDU	Variance	BMDL_COV	BMDU_COV	Variance	Expo Time
Pig										
	Kidney	DON	1	n/o	n/o	n/o	Single Expo	Single Expo	Single Expo	Single Expo
	Liver	DON	1	n/o	n/o	n/o	Single Expo	Single Expo	Single Expo	Single Expo
Chicken										
	BWG	DON	1	n/o	n/o	n/o	n/o	n/o	n/o	7
	BWG	DON	1	n/o	n/o	n/o	n/o	n/o	n/o	14
	BWG	DON	1	n/o	n/o	n/o	n/o	n/o	n/o	21
	BWG	AF	1	0.0269	0.0887	0.004743	Single Expo	Single Expo	Single Expo	Single Expo
	BWG	AF	2	0.0063	0.421	0.2928	0.47	Inf	0.05107	7
	BWG	AF	2				0.011	0.248	0.03892	14
	BWG	AF	2				0.00112	0.0619	0.0375	21
	BWG	AF	3	0.0758	0.532	0.1064	0.0897	0.345	0.07477	28
	BWG	AF	3				0.156	0.479	0.02262	49
	BWG	AF	4	0.714	1.37	0.01967	Single Expo	Single Expo	Single Expo	Single Expo
	BWG	OTA	1	0.227	1.17	0.08722	0.274	1.02	0.04833	28
	BWG	OTA	1				0.433	1.08	0.01893	49
	Liver	AF	1	0	0.459	0.03701	Single Expo	Single Expo	Single Expo	Single Expo
	Liver	AF	2	0.688	1.28	0.136	Single Expo	Single Expo	Single Expo	Single Expo
	Liver	AF	3	0.00574	0.599	0.05346	0.293	Inf	0.009215	7
	Liver	AF	3				0.138	Inf	0.01222	14
	Liver	AF	3				0.00313	0.523	0.06229	21
	Liver	AF	4	0.0441	0.351	0.03798	Single Expo	Single Expo	Single Expo	Single Expo
	Liver	OTA	1	0.101	2.79	0.05308	Single Expo	Single Expo	Single Expo	Single Expo
	Kidney	AF	1	0.0177	0.484	0.1244	0.00289	Inf	0.03541	7
	Kidney	AF	1				0.000186	0.152	0.03218	14
	Kidney	AF	1				0.0000454	0.0687	0.1658	21

Kidney	AF	2	n/o	n/o	n/o	Single Expo	Single Expo	Single Expo	Single Expo
Kidney	OTA	1	0.623	1.71	0.03022	Single Expo	Single Expo	Single Expo	Single Expo

n/o = no model output. Single Expo = data with a single exposure time.

3.8.2. Human case study: risk assessment of multiple mycotoxins using component-based and provisional daily intake approaches

The present human case study (HCS) for the risk assessment of multiple mycotoxin integrates the results of the data collection combining the contribution of the MYCHIF consortium work (namely the occurrence and co-occurrence data, the toxicological data and the biomarker of exposure data). The information on environmental, ecological, and agronomic factors affecting the relative abundance of co-occurring mycotoxins in the contaminated crops, as well as (co-)occurrence data and toxicity of multiple mycotoxin investigated, was collected and used to develop the current case study on risk assessment for humans exposed to co-occurring mycotoxins.

This case study follows the harmonised framework for risk assessment of combined exposure to multiple chemicals that consist of well-defined steps: (i) problem formulation, (ii) exposure assessment, (iii) hazard assessment, and (iv) risk characterisation. In the context of this framework, two different approaches were investigated, namely (i) the component-based approach (CBA) and (ii) the provisional daily intake (PDI).

The CBA is a procedure applied for the risk assessment of mixtures when the exposure levels and effect data of their components are known. The individual components of the mixture are organised into assessment groups (AG) following grouping criteria (e.g. physicochemical properties, hazard characteristics, exposure considerations). The dose addition is used as default model, and predictions of combined toxicity of compounds of the AG are measured from data of the toxicity of the individual components (EFSA, 2013a, 2019).

The PDI approach models the internal dose information with the available human biomarker data to derive exposure to the mixture. Several authors have performed this exercise obtaining estimated PDI, also defined by other authors as estimated daily intake (EDI), for single mycotoxins and have published a number of peer-reviewed papers (Warth et al., 2013; Ezekiel et al., 2014; Solfrizzo et al., 2014; Heyndrickx et al., 2015; Al-Jaal et al., 2019).

Problem formulation. The occurrence and co-occurrence evidences gathered in the literature led to the identification of two mycotoxin mixtures (selected among *Fusarium* mycotoxins), belonging to a group of contaminants to which humans are exposed on a chronic basis, mainly from cereal food sources (i.e. mixture-1: DON, FBs and ZEN, and mixture-2: T2/HT2, DON and NIV). Exposure and hazard data of the individual components of the two identified mixtures were collected.

Exposure assessment. The conventional dietary exposure assessment paradigm uses consumption and occurrence data to derive exposure scenarios of groups of populations to a single mycotoxin between two extreme intake values, i.e. the lower bound (LB) and upper bound (UB), for the percentile 50 (P50) and a high percentile (most frequently the P95). In the CBA, the (co-)occurrence and consumption data of cereal-food based products for each single mycotoxin were combined to obtain an individual mycotoxin exposure (Exp_i) and then summed up to obtain the total exposure, under the dose addition assumption. In the context of the PDI approach, the data of BM measured in specimen for mixture-1 were used to define the exposure estimate for the mixture. The PDI (expressed as µg of mycotoxin per kg bw per day) was estimated by combining i) the mycotoxin concentration in urine, ii) the available excretion rate for each of the mycotoxin in the mixture, iii) the human body weight (bw) and iv) the daily urine excretion volume (µg/L mycotoxin, L urine in 24 h, % excretion rate, kg bw, respectively). The PDI was calculated both for single components and for the mixture.

Hazard assessment. Hazard information were collected for the individual mycotoxins of mixture-1 and mixture-2 from toxicity studies, EFSA opinions and the chemical hazard database called OpenFoodTox (e.g. toxicity data, reference point (RP) or point of departure (POD) from chronic studies in test species (i.e. rat, mouse, pig), and reference values (TDI)).

Risk Characterisation. In the context of CBA, an equivalent factor approach is proposed using simply the RPs as conservative estimates. The Equivalent Factor (EF) of each mycotoxin of the mixture (EF_i) was calculated dividing the POD of the identified index compound (POD_{index}) (i.e. the most potent compound) by

the POD of each individual mycotoxin (POD$_i$). Dose addition is applied using a margin of exposure approach (MOE). Being the considered mycotoxins neither genotoxic nor carcinogenic, a value of 100 has been chosen as a reference/cut-off value: MOE superior to a 100-fold was interpreted as a scenario of low concern for compounds that are not genotoxic and carcinogenic whereas a 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. 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 (MOA). Since all this may lead to under or over estimation, a thorough scrutiny of the approach with the application of an uncertainty factor would be appropriate.

In PDI, the Hazard exposure index (HI) is proposed, defined as the sum of the ratio between the exposure values of each component and the respective reference value (i.e., TDI), and the resulting value was interpreted to understand the acceptability of the risk.

3.8.2.1. Component based approach

The CBA is presented as an exploratory case study of human risk assessment of combined exposure to multiple mycotoxins in food. In the tiered approach, the human risk assessment of combined exposure is explored considering exposure and effect data of the individual mycotoxin. In the present human case study, the following was considered:

- *Cereals and cereal-based food diet, aggregate chronic exposure assessment.*

- *Occurrence, co-occurrence for cereal commodities.* Occurrence and co-occurrence data were extracted from MYCHIF database (all cereals-based food), which include all data collected in EU countries in food samples and accounts only for records reporting MeanTot value or MeanPos value or Concentration value (available online on the MYCHIF repository and EFSA knowledge junction (10.5281/zenodo.3615174)). In absence of these values, when Min and Max and % of samplings >LOD values were reported, the median values were considered. Two datasets, one based on occurrence data and one on co-occurrence data, were made available and used separately as input for the model.

- *Consumption data for the specific cereal commodities.* EFSA Comprehensive European Food Consumption Database was used for extracting data of FoodEx2 at level 1 associated to consumers' only and mean and high consumers (95th percentile consumption). Exposure data were calculated following the equation (eq. 1):

$$EXP_i = [\text{consumption (g/day)} / 1000] * 1/kg_{bw} * \text{mycotoxin}_{Occ/Co-occ} \text{ (µg/kg)} \qquad \text{(eq. 1)}$$

where:

consumption (g/day), are mean and P95 consumption values. Data were extracted from EFSA Foodex2-level-1 (Grains and grain-based products) for adolescent, adult and elderly

mycotoxin$_{Occ/Co-occ}$ (µg/kg), are data extracted from MYCHIF database for all cereals-based food, when mycotoxin occurred as single compound or co-occurred in a group.

Exposure estimates were calculated for three scenarios: minimum Lower Bound (LB), medium Lower Bound (MB) and maximum Upper Bound (UB) in µg/kg bw per day. For each substance, the three scenarios were obtained by substituting LOD=0 and LOQ=LOD in the LB, LOD=0.5*LOD and LOQ=LOQ*0.5 in MB and LOD=LOD and LOQ=LOQ in the UB (EFSA, 2010b; Ingenbleek et al., 2019).

- *Hazard and Equivalent Factors (EFs).* Toxicological end points as PODs (such as BMDL or NOAEL) have been extracted from the EFSA CONTAM opinions (tracing author, year, study, test type, species, endpoint, value, unit, effect, toxicity) for each mycotoxin. EFs were calculated taking as the identified

index compound (POD_{index}) the value of the most potent compound for which the EF_{index} is considered =1. The EF_i of each mycotoxin is then calculated with the following equation (eq. 2)

$$EF_i = POD_{index} / POD_i \qquad \text{(eq. 2)}$$

- *Risk characterization*. As far as the risk characterization, the margin of exposure (MOE) was estimated (eq. 3), as the ratio between the POD of the index compound (the most potent compound) and the sum of normalised individual exposures $Sum(Exp_i)*(EF_i)$.

$$MOE = POD_{index} / Sum(Exp_i)*(EF_i) \qquad \text{(eq. 3)}$$

Being the three considered mycotoxins neither genotoxic nor carcinogenic, a value of 100 has been chosen as a reference/cut-off value: MOE values above 100 do not raise human health concern, values below 100 suggest a scenario of concern.

It is recognised that all values below 100 indicate a signal to proceed to a higher tier, with the possible need to adjust the approach with additional data such as those deriving from animal data analysis.

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 MOA. Since all this may lead to under or over estimation, a thorough scrutiny of the approach with the application of an uncertainty factor would be appropriate.

Mixture-1: DON, FBs and ZEN

<u>Problem formulation</u>. The harmonised framework is applied for the human risk assessment of the mixture-1 (consisting in DON, FBs and ZEN) from cereal-based food sources. The risk assessment is performed for European consumers. Each mycotoxin of the AG is well characterized for its structure, the chronic exposure levels in food are assessed for each single mycotoxin, the hazard properties are defined and were derived from assessment studies as listed in Table 24. The assessment group was set based on the (co)-exposure as the three compounds can reasonably occur together in food commodities.

<u>Hazard identification and characterization</u>. For DON and FBs, hazard characterisation was performed using benchmark dose lower confidence limits for 5 and 10% of effect (BMDL5 and BMDL10) as PODs (110 and 100 µg/kg bw/day for DON and FBs, respectively). They were based on reduced feed intake and reduced body weight gain for DON and on non-neoplastic hepatotoxicity in mice with a critical effect associated with an increased incidence of megalocytic hepatocytes in the liver (histopathology) for FBs. For ZEN, hazard characterisation was performed using a NOAEL of 1000 µg/kg bw per day based on an increased incidence of hepatocellular cytoplasmic vacuolization in male rats. PODs were extracted from EFSA assessments. Relative equivalent factors were calculated using the FBs as the index compound (most potent compound) Table 25. EFs for each substance were calculated as in equation 2. No evidence of interactions between the compounds was available from the literature.

The summary of hazard data is listed in Table 24. PODs (µg/kg bw per day) and calculated EFs are shown in Table 25.

Table 24: Hazard data. Points of departure

Mycotoxin	Author	Year	Test Type	Species	Endpoint	Value	Unit	Effect	Toxicity
DON	EFSA CONTAM	2017	chronic	Mouse	BMDL05	110	µg/kg bw/day	body weight	systemic

| FBs | EFSA CONTAM | 2018 | chronic | Mouse | BMDL10 | 100 | µg/kg bw/day | histopathology non neoplastic | hepatotoxicity |
| ZEN | EUROM IX case study (2019) | | chronic | Rat | NOEL | 1000 | µg/kg bw/day | hepatocellular cytoplasmatic vacuolization | hepatotoxicity |

Table 25: Equivalent Factors

Mycotoxin	Point of departure (POD) (µg/kg bw per day)	Equivalent Factor (EF)
DON	$BMDL_5$ = 110	0.91
FB	$BMDL_{10}$ = 100	1
ZEN	NOEL = 1000	0.10

Exposure. Exposure assessment was performed as the product between occurrence data and consumption data. In particular, occurrence of DON, FBs, ZEN (and their modified forms) were extracted from the MYCHIF database (all cereals-based food category, for more details see 3.4), both as occurrence of each single mycotoxin as well as co-occurrence of the three mycotoxins. These data were extracted for each country and aggregated at EU level. Then, αZEL and βZEL concentrations were corrected on the basis of their Potency Factors (i.e. PF: 60 for αZEL, 0.2 for βZEL).

Consumption data were gathered from EFSA Comprehensive European Food Consumption Database. In order to estimate the chronic dietary exposure to cereal products, consumption data for average and high consumers (P95) were extracted for cereal commodities at Foodex2-level-1 (i.e. Grains and grain-based products) and aggregated at EU level. The exposures were estimated for average and high consumers at LB/UB (µg/kg bw per day) in three subpopulations, namely 'Adolescents' (≥ 10 years to < 18 years old), 'Adults (≥ 18 years to < 65 years old) and 'Elderly' (≥ 65 years to < 75 years old). Exposure estimates were calculated using either occurrence data of single mycotoxins and co-occurrence data (

Table **26**). Table 27 reports the P95 at LB and UB of the normalised exposure $(Exp_i)*(EF_i)$ obtained for each of the mycotoxins considered in occurrence and co-occurrence conditions. After summing each normalised exposure $(Exp_i)*(EF_i)$, the UB and LB range of combined dose addiction was defined for the two occurrence conditions.

Risk characterization. The risk characterization was carried out comparing the sum of normalised individual exposures $(Sum(Exp_i)*(EF_i))$ with the POD_{index} of the index compound (i.e. FB). The MOE was calculated as the ratio between the POD_{FB} and the sum of the individual exposures, as follows (eq. 7)

$$MOE = POD_{FB} / Sum(Exp_i)*(EF_i) \quad (eq.\ 7)$$

Table 27 also reports the MOE values obtained for both occurrence and co-occurrence conditions.

MOE superior to a 100-fold was interpreted as a scenario of low concern for compounds that are not genotoxic and carcinogenic whereas a 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. All values resulted in values below 100. To noted that as a critical approximation, the grouping principle is not based on the same MOA.

Uncertainty in the hazard assessment step is dealing with the miscellaneous of endpoints derived from diverse modes of action, and the lack of data on potential interaction among the considered mycotoxins (i.e. synergistic/additive or antagonistic effect). This may lead on under- or over- estimate the risk of multiple mycotoxins, therefore the application of an uncertainty factor would be appropriate.

A reporting table summarizing the human risk assessment of combined exposure to the mixtures, is shown in Table 36.

Table 26: Exposure estimates for single mycotoxins and for the mixture

Mycotoxin	Exposure (occurrence) µg/kg bw/d LB		Exposure (co-occurrence) µg/kg bw/d LB		Exposure (occurrence) µg/kg bw/d UB		Exposure (co-occurrence) µg/kg bw/d UB	
	Mean	P95	Mean	P95	Mean	P95	Mean	P95
DON	1.333	2.458	0.871	1.607	1.412	2.605	0.992	1.830
FB	0.783	1.444	0.569	1.049	0.849	1.566	0.734	1.355
ZEN	0.317	0.585	0.675	1.245	0.614	1.133	1.030	1.900
	2.433	**4.487**	**2.115**	**3.901**	**2.875**	**5.304**	**2.756**	**5.085**

Table 27: Normalised exposure (Expi)*(EFi) and MOE values

Compound	Hazard Metrics	$(Exp_i)*(EF_i)$ (occurrence) µg/kg bw/d		$(Exp_i)*(EF_i)$ (co-occurrence) µg/kg bw/d		$Sum(Exp_i)*(EF_i)$ (occurrence) µg/kg bw/d		$Sum(Exp_i)*(EF_i)$ (co-occurrence) µg/kg bw/d		MOE (occurrence)		MOE (co-occurrence)	
	EF	LB P95	UB P95	LB P95	UB P95	LB	UB	LB	UB	LB	UB	LB	UB
DON	0.91	2.2349	2.3682	1.4605	1.6635	3.74	4.05	2.63	3.21	26.8	24.7	38.0	31.2
FB	1	1.4442	1.5657	1.0492	1.3547								
ZEN	0.10	0.0585	0.1133	0.1245	0.1900								
		3.7376	4.0472	2.6341	3.2082								

Mixture-2: T2/HT2, DON and NIV

Problem formulation. The harmonised framework is applied for the human risk assessment of the mixture-2 (consisting in T2/HT2, DON and NIV) from cereal-based food sources. The risk assessment is performed for European consumers. Each mycotoxin of the group is well characterized for its structure, the chronic exposure levels in food are assessed for each single mycotoxin, the hazard properties are defined and were derived from assessment studies as listed in Table 28. The assessment group was set on the basis that the mixture-2 can occur in food commodities.

Hazard identification and characterization. For all the mycotoxins of mixture-2, hazard characterisation was performed using benchmark dose lower confidence limits for 5 and 10% of effect (BMDL5 and BMDL10), so as PODs were 110, 350 and 3.33 µg/kg bw/day for DON, NIV and T2/HT2, respectively. They have been based on reduced feed intake and reduced body weight gain in the case of DON, on a reduction of total leukocyte count in in-vivo subchronic toxicity study for T2/HT2 and on reduced white blood cell counts observed in a 90-day rat study for NIV. PODs have been extracted from EFSA assessments (Table 28). Relative equivalent factors were calculated using the FBs as the index compound (most potent compound) Table 29. EFs for each substance were calculated as in equation 2. No evidence of interactions between the compounds was available from the literature.

Exposure. Occurrence data and co-occurrence data used for the exposure calculations were extracted from the MYCHIF database (all cereals-based food). Occurrence on DON, NIV, T2/HT2 and their masked forms were extracted for each EU country (sampCountry), including all data collected in EU countries in food samples and accounting only for records reporting MeanTot value or MeanPos value or Concentration values in. In absence of these values, the median values were also included when Min and Max and % of samplings >LOD values were reported. Two datasets, one based on occurrence data and one on co-occurrence data, were then prepared and used separately as input for the model. Consumption data were taken from EFSA Foodex2-level-1 (Grains and grain-based products, mean and 95th percentile consumption in total population). Countries were clustered in eight macro areas (Battilani et al., 2012) taking into account both climate characteristics and latitude as indicated in Table 30. For each considered macro area, LB, MB, and UB exposure values were calculated for adolescent, adult and elderly including both consumption scenarios (i.e., mean and P95).

Figure 34, Figure 36 and Figure 38 present a visual analysis of the co-occurrence data available for each involved EU country. For each mycotoxin (i.e., DON, T2/HT2 and NIV) average values, number of records, min and max were reported. Figure 35, Figure 37 and Figure 39 present a visual analysis of the exposure calculated for each mycotoxin (co-occurrence dataset) at macro-area level for adults, MB scenario and mean consumption value. Figure 40, Figure 42 and Figure 44 present a visual analysis of the occurrence data available for each EU country. For each mycotoxin (DON, T2/HT2 and NIV) average values, number of records, min and max were reported. Figure 41, Figure 43 and Figure 45 present a visual analysis of the exposure calculated for each mycotoxin (occurrence dataset) at macro-area level for adults, MB scenario and mean consumption value.

Table 31 (a, b, c) summarizes the LB and UB P95 exposure estimates for either occurrence or co-occurrence conditions for adolescent, adult and elderly.

Table 32 (a, b, c) reports the P95 at LB and UB range of the normalised exposure $(Exp_i)*(EF_i)$ obtained for each one of the mycotoxins considered in occurrence and co-occurrence conditions.

After summing each normalised exposure $(Exp_i)*(EF_i)$, the UB and LB range of combined dose addiction was defined for the two occurrence conditions. Table 32 reports values for adolescents, adults and elderly.

Risk characterization. The risk characterization was carried out comparing the sum of normalised individual exposures (combined dose addiction, $Sum(Exp_i)*(EF_i)$) with the POD_{index} of the index compound (i.e., T2/HT2) (see summary in Table 33). The MOE was calculated as the ratio between the POD_{index} (T2/HT2) and the sum of the individual exposures $Sum(Exp_i)*(EF_i)$, by using (eq. 3).

Table 34 summarizes the MOE values obtained for the group of populations considered (adolescents, adults and elderly), for both occurrence and co-occurrence conditions. The whole picture of values shows a great heterogeneity. Geographical area 5 shows values >100 for adolescents and <100 for adults, while geographical area 6 shows the opposite scenario (<100 for adolescents and >100 for adults). All the other geographical areas report values <100. On the other hand, when the calculations are done for ALL, MOE values are far below 100. It should be noted that the most critical approximation is for the grouping principle that is not based on the similarity of the MOA.

In addition, the uncertainty in the hazard assessment step deals with what derived from diverse MOA (despite the use of same end reference points), and with the lack of data on potential interaction among the considered mycotoxins (i.e. synergistic/additive or antagonistic effect) (see Table 35). It should be noted that values below 100 indicate a signal to proceed to a higher tier, with the possible need to adjust the approach with additional data. A table summarizing the risk assessment of combined exposure to multiple mycotoxins, as scrutinized in these examples, is shown in Table 36.

Table 28: Hazard data. Points of departure

Mycotoxin	Author	Year	Study	Test Type	Species	Endpoint	Value	Unit	Effect	Toxicity
DON	EFSA CONTAM	2017	Human health	chronic	Mouse	BMDL05	110	µg/kg bw/day	Body weight	systemic
NIV	EFSA CONTAM	2013	Human health	subchronic	Rat	BMDL05	350	µg/kg bw/day	Immunology	immunotoxicity
T2+HT2	EFSA CONTAM	2017	Human health	subchronic		BMDL10	3.33	µg/kg bw/day	Haematology	hemopoietic

Table 29: Equivalent Factors

Mycotoxin	Point of departure (POD) (µg/kg bw per day)	Equivalent Factor (EF)
DON	$BMDL_5 = 110$	0.03
NIV	$BMDL_5 = 350$	0.0095
T2+HT2	$BMDL_{10} = 3.33$	1

Table 30: Macroarea clusters

Macroarea		Countries
1	BALKGREE	Balkans countries, Bulgaria, Greece, Cyprus
2	BALTIC	Estonia, Latvia, Lithuania
3	EEUROPA	Slovakia, Hungary, Romania, Poland
4	ENG	Ireland and United Kingdom
5	IBERIA	Spain and Portugal
6	ITALIA	Italy, Malta
7	MIDDLEEU	France, Switzerland, Austria, Belgium, Netherlands, Germany, Czech Rep, Denmark
8	SCAND	Norway, Sweden, Finland

Table 31 a, b, c: Exposure estimates for adolescents, adults and elderly

a) Mycotoxin	Adolescents - Exposure (µg/kg bw/d) LB P95									Adolescents - Exposure (µg/kg bw/d) UB P95								
	Area									Area								
	1	2	3	4	5	6	7	8	ALL	1	2	3	4	5	6	7	8	ALL
DON (Co-occ)	1.712			2.603	0.270	7.514	0.799		1.956	1.712			2.603	0.628	7.818	0.903		2.228
DON (Occ)	0.844	1.146		0.643	2.203	0.782	4.229	5.229	2.994	1.052	1.146		0.729	2.303	1.023	4.503	5.242	3.172
NIV (Co-occ)				0.686	0.062	0.105			0.273				0.686	0.072	0.209			0.530
NIV (Occ)				0.313	0.000		0.447	0.425	0.354				0.318	0.441		0.500	0.442	0.436
T2+HT2 (Co-occ)				2.567	0.018	0.027	0.094		0.433				2.567	0.029	0.039	0.143		0.514
T2+HT2 (Occ)	0.175	0.085		0.059	0.055		0.187	0.149	0.164	0.211	0.085		0.059	0.207		0.240	0.169	0.207

b) Mycotoxin	Adults - Exposure (µg/kg bw/d) LB P95									Adults - Exposure (µg/kg bw/d) UB P95								
	Area									Area								
	1	2	3	4	5	6	7	8	ALL	1	2	3	4	5	6	7	8	ALL
DON (Co-occ)	1.695		0.768	2.060	0.162	5.030	0.577		1.406	1.695		1.240	2.060	0.377	5.233	0.653		1.602
DON (Occ)	0.836	0.712	1.132	0.509	1.320	0.523	3.058	5.117	2.152	1.041	0.712	1.164	0.577	1.380	0.685	3.256	5.129	2.281
NIV (Co-occ)			0.152	0.543	0.037	0.070			0.196			0.545	0.543	0.043	0.140			0.381
NIV (Occ)			0.000	0.248	0.000		0.323	0.416	0.255			0.052	0.252	0.264		0.361	0.432	0.314
T2+HT2 (Co-occ)			0.014	2.031	0.011	0.018	0.068		0.311			0.178	2.031	0.018	0.026	0.103		0.369
T2+HT2 (Occ)	0.173	0.053	0.054	0.046	0.033		0.135	0.145	0.118	0.209	0.053	0.069	0.046	0.124		0.173	0.165	0.149

c) Mycotoxin	Elderly - Exposure (µg/kg bw/d) LB P95									Elderly - Exposure (µg/kg bw/d) UB P95								
	Area									Area								
	1	2	3	4	5	6	7	8	ALL	1	2	3	4	5	6	7	8	ALL
DON (Co-occ)			0.783	2.046	0.131	4.571	0.513		1.270			1.265	2.046	0.305	4.755	0.580		1.447
DON (Occ)		0.576	1.155	0.506	1.068	0.476	2.718	4.880	1.944		0.576	1.187	0.573	1.117	0.623	2.894	4.892	2.060

Mycotoxin	1	2	3	4	5	6	7	8	ALL	1	2	3	4	5	6	7	8	ALL
NIV (Co-occ)			0.155	0.539	0.030	0.064			0.177			0.555	0.539	0.035	0.127			0.344
NIV (Occ)			0.000	0.246	0.000		0.288	0.397	0.230			0.053	0.250	0.214		0.321	0.412	0.283
T2+HT2 (Co-occ)			0.015	2.017	0.009	0.016	0.061		0.281			0.181	2.017	0.014	0.024	0.092		0.334
T2+HT2 (Occ)	0.042		0.055	0.046	0.026		0.120	0.139	0.106	0.042		0.071	0.046	0.100		0.154	0.157	0.134

Table 32 a, b, c: Normalised exposure (Expi)*(EFi) for adolescents, adults and elderly

a) Mycotoxin

Mycotoxin	Adolescents - (Expi)*(EFi) LB P95									Adolescents - (Expi)*(EFi) UB P95								
	Area									Area								
	1	2	3	4	5	6	7	8	ALL	1	2	3	4	5	6	7	8	ALL
DON (Cco-occ)	0.051			0.078	0.008	0.225	0.024		0.059	0.051			0.078	0.019	0.235	0.027		0.067
DON (Occ)	0.025	0.034		0.019	0.066	0.023	0.127	0.157	0.090	0.032	0.034		0.022	0.069	0.031	0.135	0.157	0.095
NIV (Co-occ)				0.007	0.001	0.001			0.003				0.007	0.001	0.002			0.005
NIV (Occ)				0.003	0.000		0.004	0.004	0.003				0.003	0.004		0.005	0.004	0.004
T2+HT2 (Co-occ)				2.567	0.018	0.027	0.094		0.433				2.567	0.029	0.039	0.143		0.514
T2+HT2 (Occ)	0.175	0.085		0.059	0.055		0.187	0.149	0.164	0.211	0.085		0.059	0.207		0.240	0.169	0.207

b) Mycotoxin

Mycotoxin	Adults - (Expi)*(EFi) LB P95									Adults - (Expi)*(EFi) UB P95								
	Area									Area								
	1	2	3	4	5	6	7	8	ALL	1	2	3	4	5	6	7	8	ALL
DON (Cco-occ)	0.051		0.023	0.062	0.005	0.151	0.017		0.042	0.051		0.037	0.062	0.011	0.157	0.020		0.048
DON (Occ)	0.025	0.021	0.034	0.015	0.040	0.016	0.092	0.153	0.065	0.031	0.021	0.035	0.017	0.041	0.021	0.098	0.154	0.068
NIV (Co-occ)			0.001	0.005	0.000	0.001			0.002			0.005	0.005	0.000	0.001			0.004
NIV (Occ)			0.000	0.002	0.000		0.003	0.004	0.002			0.000	0.002	0.003		0.003	0.004	0.003
T2+HT2 (Co-occ)			0.014	2.031	0.011	0.018	0.068		0.311			0.178	2.031	0.018	0.026	0.103		0.369

	1	2	3	4	5	6	7	8	ALL	1	2	3	4	5	6	7	8	ALL
T2+HT2 (Occ)	0.173	0.053	0.054	0.046	0.033	0.000	0.135	0.145	0.118	0.209	0.053	0.069	0.046	0.124	0.000	0.173	0.165	0.149

c) Mycotoxin	Elderly - (Expi)*(EFi) LB P95										Elderly - (Expi)*(EFi) UB P95								
	Area										Area								
	1	2	3	4	5	6	7	8	ALL	1	2	3	4	5	6	7	8	ALL
DON (Cco-occ)			0.024	0.061	0.004	0.137	0.015		0.038			0.038	0.061	0.009	0.143	0.017		0.043
DON (Occ)		0.017	0.035	0.015	0.032	0.014	0.082	0.146	0.058		0.017	0.036	0.017	0.033	0.019	0.087	0.147	0.062
NIV (Co-occ)			0.001	0.005	0.000	0.001			0.002			0.005	0.005	0.000	0.001			0.003
NIV (Occ)			0.000	0.002	0.000		0.003	0.004	0.002			0.001	0.002	0.002		0.003	0.004	0.003
T2+HT2 (Co-occ)			0.015	2.017	0.009	0.016	0.061		0.281			0.181	2.017	0.014	0.024	0.092		0.334
T2+HT2 (Occ)		0.042	0.055	0.046	0.026		0.120	0.139	0.106		0.042	0.071	0.046	0.100		0.154	0.157	0.134

Table 33: LB and UB range of combined dose addiction (Expi)*(EFi)

Mycotoxin	(Expi)*(EFi) LB P95										(Expi)*(EFi) UB P95								
	Area										Area								
	1	2	3	4	5	6	7	8	ALL	1	2	3	4	5	6	7	8	ALL
Sum(Expi)*(EFi) (Co-occurrence) Adolescents	0.05			2.65	0.03	0.25	0.12		0.49	0.05			2.65	0.05	0.28	0.17		0.59
Sum(Expi)*(EFi) (Occurrence) Adolescents	0.20	0.12		0.08	0.12	0.02	0.32	0.31	0.26	0.24	0.12		0.08	0.28	0.03	0.38	0.33	0.31
Sum(Expi)*(EFi) (Co-occurrence) Adults	0.05		0.04	2.10	0.02	0.17	0.09		0.36	0.05		0.22	2.10	0.03	0.18	0.12		0.42

Sum(Exp$_i$)*(EF$_i$) (Occurrence) Adults	0.20	0.07	0.09	0.06	0.07	0.02	0.23	0.30	0.18	0.24	0.07	0.10	0.07	0.17	0.02	0.27	0.32	0.22
Sum(Exp$_i$)*(EF$_i$) (Co-occurrence) Elderly			0.04	2.08	0.01	0.15	0.08		0.32			0.22	2.08	0.02	0.17	0.11		0.38
Sum(Exp$_i$)*(EF$_i$) (Occurrence) Elderly		0.06	0.09	0.06	0.06	0.01	0.20	0.29	0.17		0.06	0.11	0.07	0.14	0.02	0.24	0.31	0.20

Table 34: MoE

Mycotoxin	MOE LB P95 Area									MOE UB P95 Area								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**ALL**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**ALL**
Sum(Exp$_i$)*(EF$_i$) (Co-occurrence) Adolescents	64.25			1.24	122.03	13.02	27.88		6.68	64.25			1.24	67.71	11.96	19.41		5.63
Sum(Exp$_i$)*(EF$_i$) (Occurrence) Adolescents	16.46	27.75		40.78	27.35	140.68	10.39	10.66	12.84	13.61	27.75		39.50	11.77	107.48	8.70	10.00	10.78
Sum(Exp$_i$)*(EF$_i$) (Co-occurrence) Adults	64.90		85.21	1.57	203.60	19.45	38.56		9.29	64.90		14.99	1.57	112.97	17.87	26.85		7.84
Sum(Exp$_i$)*(EF$_i$) (Occurrence) Adults	16.63	44.66	37.72	51.53	45.63	210.14	14.37	10.89	17.87	13.75	44.66	31.56	49.92	19.64	160.55	12.03	10.22	14.99
Sum(Exp$_i$)*(EF$_i$) (Co-occurrence) Elderly			83.56	1.58	251.68	21.40	43.38		10.29			14.70	1.58	139.64	19.67	30.20		8.68

| Sum(Exp$_i$)*(EF$_i$) (Occurrence) Elderly | | 55.24 | 36.99 | 51.89 | 56.41 | 231.28 | 16.16 | 11.42 | 19.78 | | 55.24 | 30.95 | 50.27 | 24.28 | 176.69 | 13.53 | 10.71 | 16.60 |

Table 35: Summary of uncertainties

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	A deterministic approach was adopted even if uncertainties of the input modeling data (mycotoxin concentration) were not negligible. Due to the scarcity of concentration data for many countries, a probabilistic approach can only be applied at EU level (as done for the PDI).
Data representativeness	Different availability and geographic distribution of mycotoxins concentrations in both dataset (occurrence and co-occurrence). Some countries, for which consumption data were not available but concentration data were available, were included in the macro area aggregation.

Table 36: Summary of the human risk assessment of combined exposure to multiple mycotoxins

Problem formulation	Description mixture	Mixture of mycotoxins, namely *Fusarium* toxins. Mixture-1 (DON, FBs and ZEA) and mixture-2 (T2/HT2, DON and NIV)
	Conceptual model	Exposure to a group of mycotoxins through food either in the case when they co-occur and occur in food Exposure pattern: chronic Common source for grouping: co-exposure Hazard data: different reference point for each compound
	Methodology	Assessment group: grouping on the basis of exposure as they can co-occur in cereal-based food commodities
	Analysis plan	Risk assessment in food for European consumers' health
Exposure assessment	CBA	Component-based approach
	Summary occurrence data	Occurrence, co-occurrence from MYCHIF database and exposure assessment for chronic exposure using EFSA Comprehensive European Food Consumption Database was used extracting data of FoodEx2 at level 1.
	Assumptions	LB and UB Highest 95th centile chronic exposure (conservative)
	Uncertainties	Maximum exposure used: overestimation of exposure
Hazard identification and hazard Characterisation	CBA	Component-based approach (CBA)-assessment group and set using the exposure as grouping criteria
	Reference points	For each component as NOAEL/ $BMDL_{10}$ from chronic studies in test species (rats etc.) EF using indexed compound (FB in mixture-1 and T2/HT2 in mixture-2)
	Combined toxicity	Dose addition
	Summary hazard metrics	Equivalent Factor (EF) for each component
	Uncertainties	Uncertainties in POD (LOEAL, NOEL, $BMDL_5$, $BMDL_{10}$ values) for each component. Use of reference points derived from diverse Mode of Action

Risk characterisation	Decision points	Margin of exposure (MOE)
	Assumptions	Dose addition
	Summary risk metrics	MOE
	Uncertainties	Uncertainties in exposure, hazard assessment (methods for exposure, human relevance of effects in rats etc.) Conservative approach
	Interpretation	MOE <100
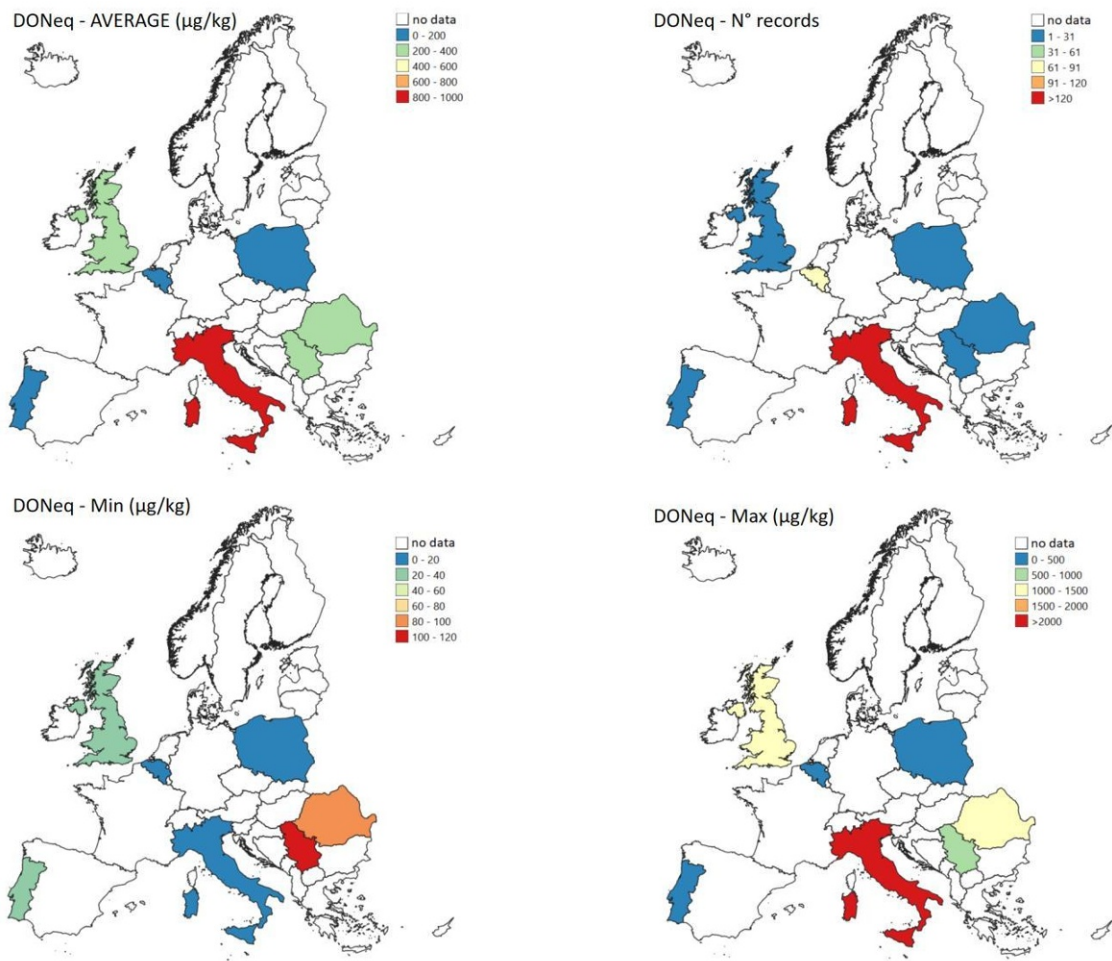
Figure 34: DON extracted from Co-occurrence dataset for each EU country. Panel upper left reports mean value of DON, panel upper right reports number of records, panel lower left min value of DON and panel lower right max value of DON.

DONeq Exposure (Adult , Medium Bound) - Co- occurrence data
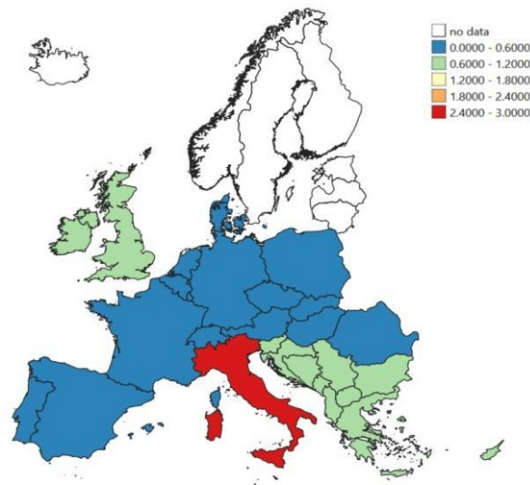
Figure 35: Exposure calculated for DON (co-occurrence dataset) at macro-area level for Adult, MB scenario and mean consumption value.
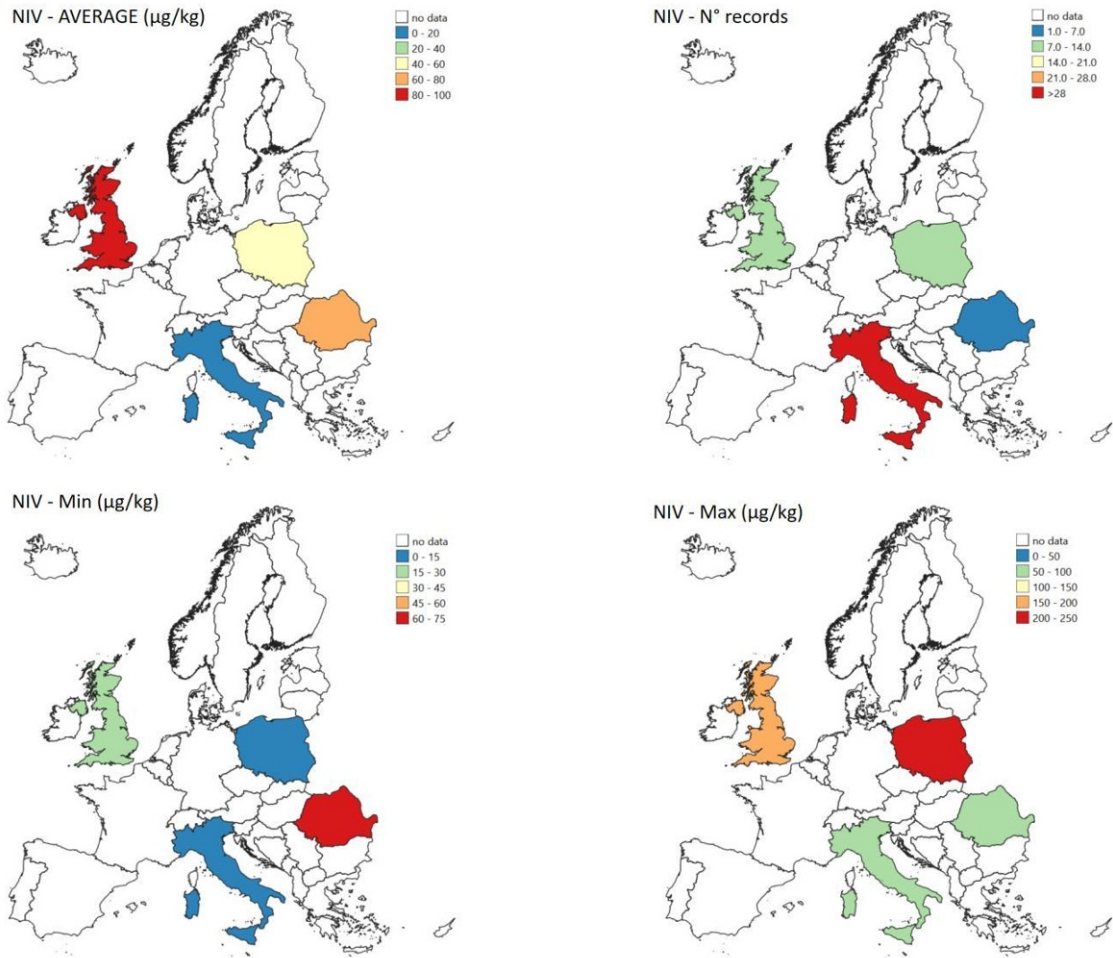
Figure 36: NIV extracted from Co-occurrence dataset for each EU country. Panel upper left reports mean value of NIV, panel upper right reports number of records of NIV, panel lower left min value of NIV and panel lower right max value of NIV.

Figure 37: Exposure calculated for NIV (co-occurrence dataset) at macro-area level for adult, MB scenario and mean consumption value.

Figure 38: T2+HT2 extracted from Co-occurrence dataset for each EU country. Panel upper left reports mean value of T2+HT2, panel upper right reports number of records of T2+HT2, panel lower left min value of T2+HT2 and panel lower right max value of T2+HT2.

T2+HT2 Exposure (Adult , Medium Bound) - Co- occurrence data

Figure 39: Exposure calculated for T2+HT2 (co-occurrence dataset) at macro-area level for Adult, MB scenario and mean consumption value.

Figure 40: DON extracted from Occurrence dataset for each EU country. Panel upper left reports mean value of DON, panel upper right reports number of records, panel lower left min value of DON and panel lower right max value of DON.
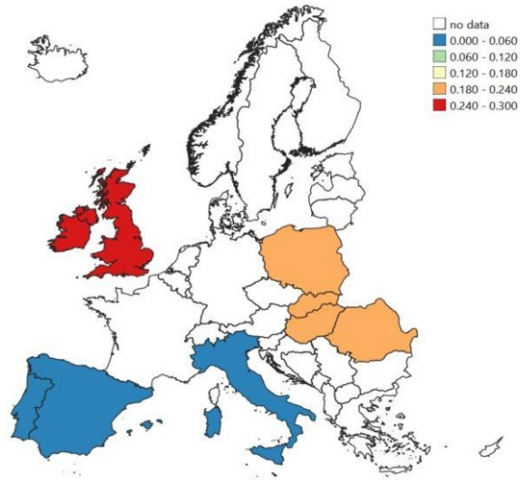
DONeq Exposure (Adult , Medium Bound) - Occurrence data

Figure 41: Exposure calculated for DON (occurrence dataset) at macro-area level for Adult, MB scenario and mean consumption value.
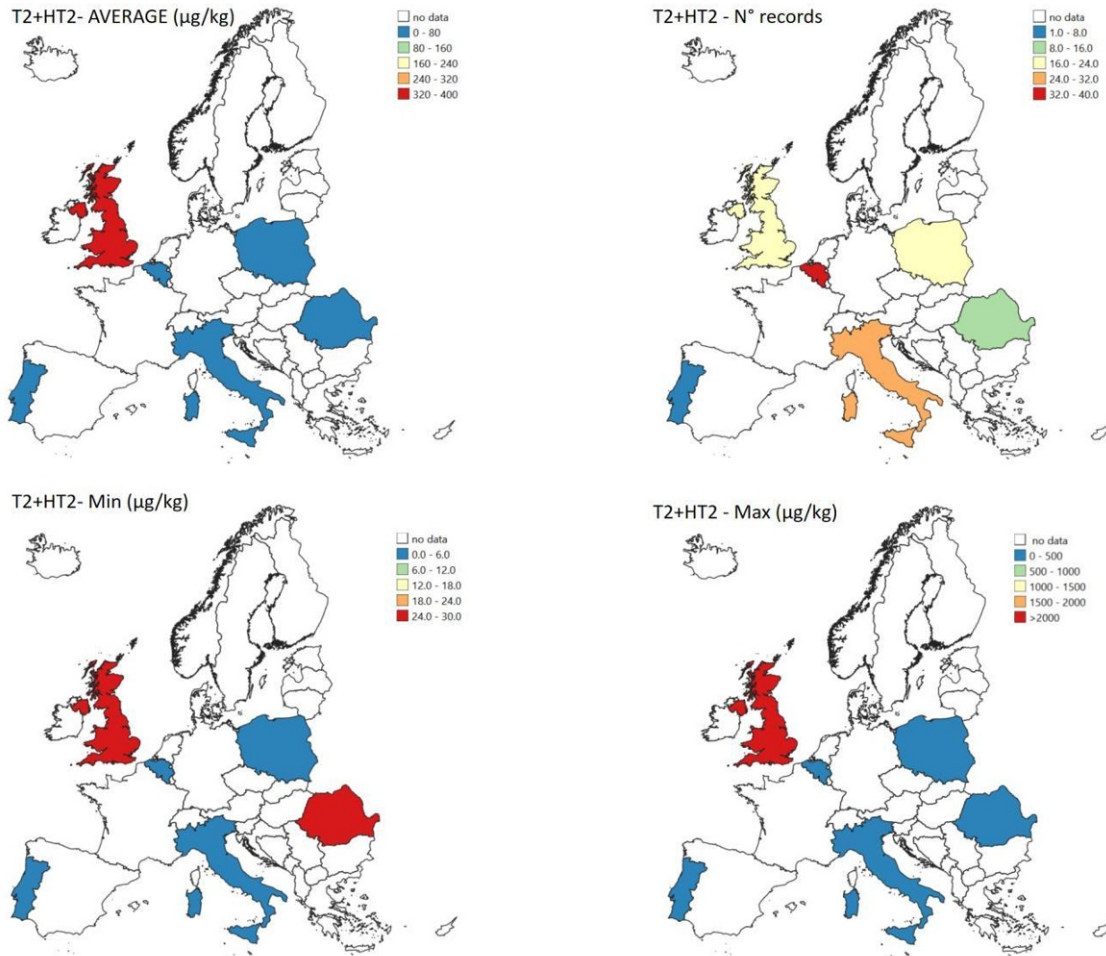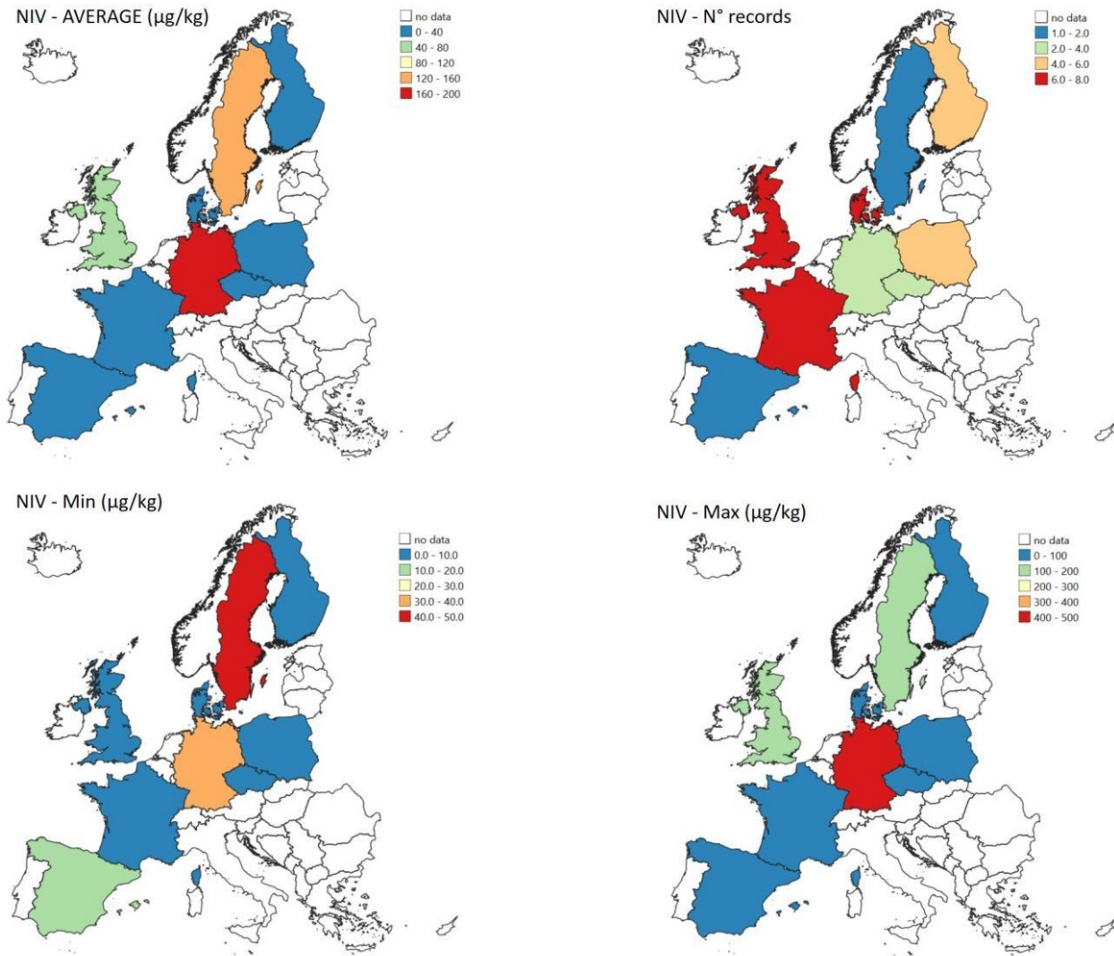
Figure 42: NIV extracted from Occurrence dataset for each EU country. Panel upper left reports mean value of NIV, panel upper right reports number of records of NIV, panel lower left min value of NIV and panel lower right max value of NIV.
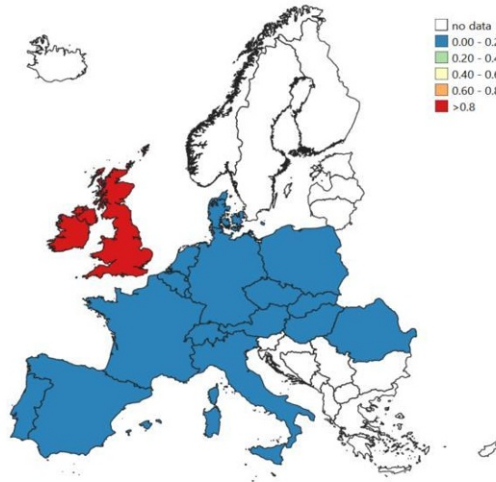
NIV Exposure (Adult , Medium Bound) - Occurrence data
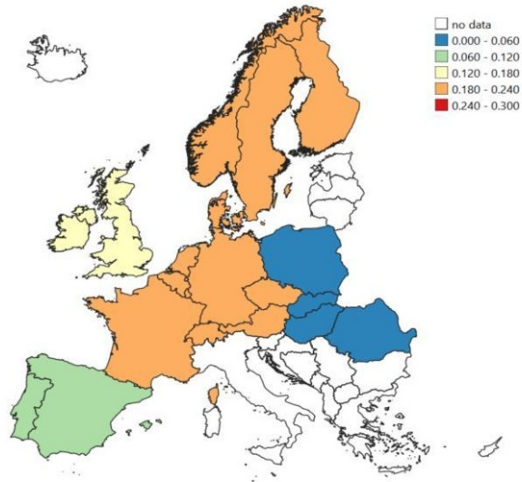
Figure 43: Exposure calculated for NIV (occurrence dataset) at macro-area level for Adult, MB scenario and mean consumption value.
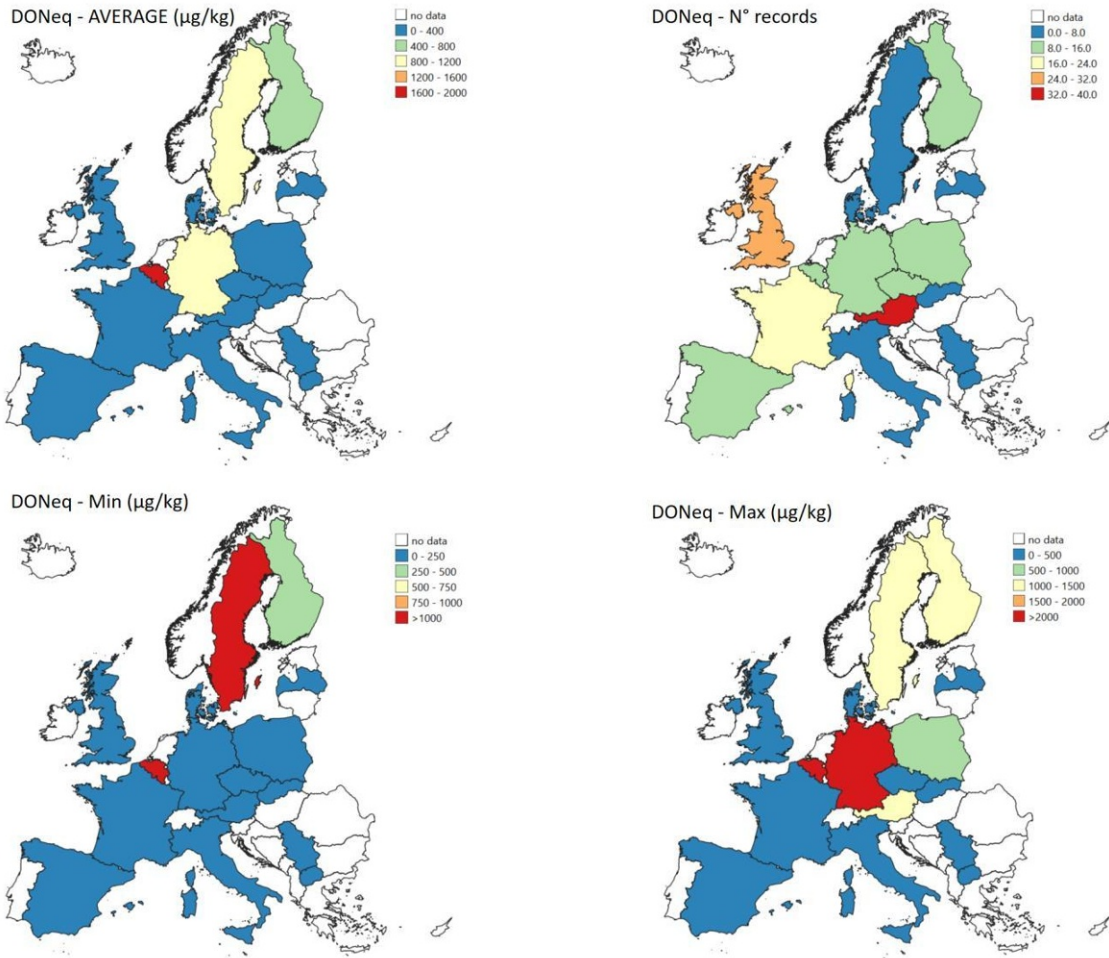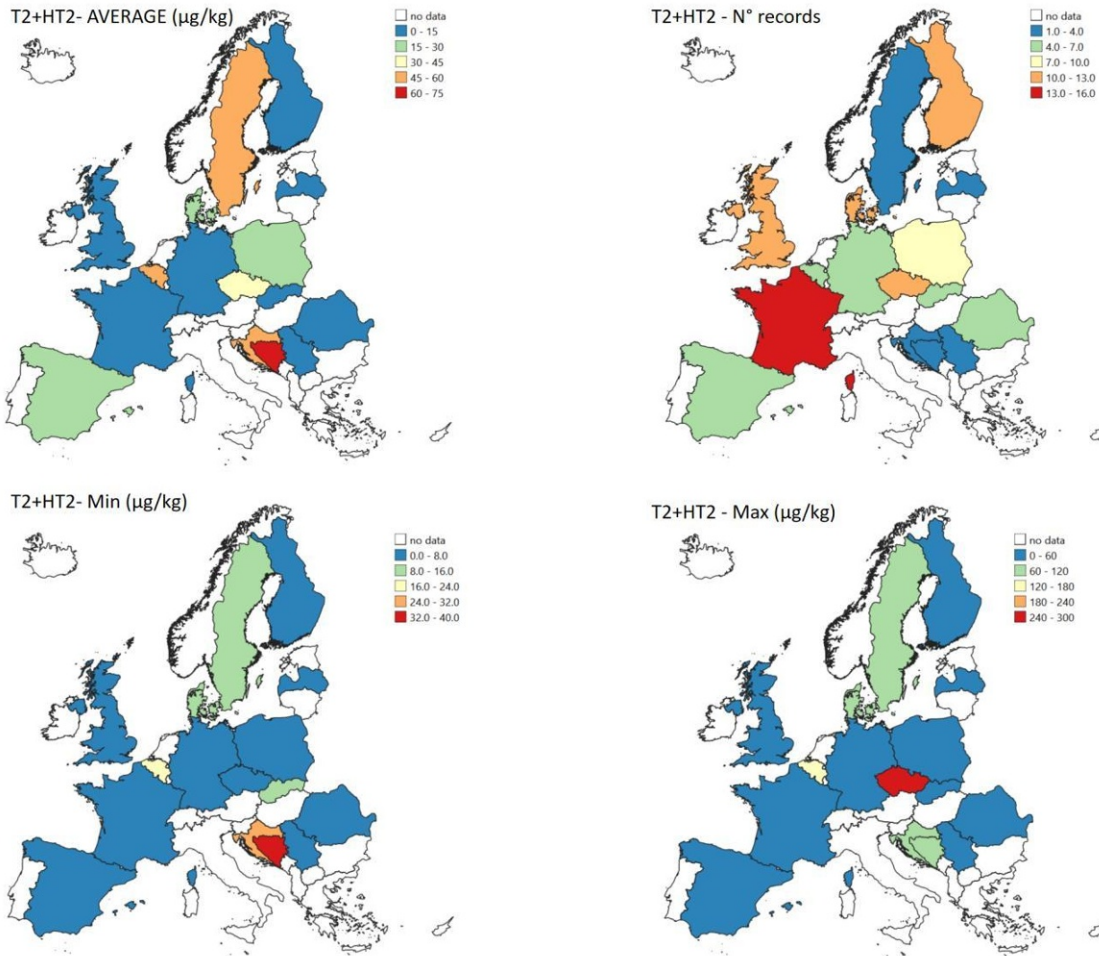
Figure 44: T2+HT2 extracted from Occurrence dataset for each EU country. Panel upper left reports mean value of T2+HT2, panel upper right reports number of records of T2+HT2, panel lower left min value of T2+HT2 and panel lower right max value of T2+HT2.
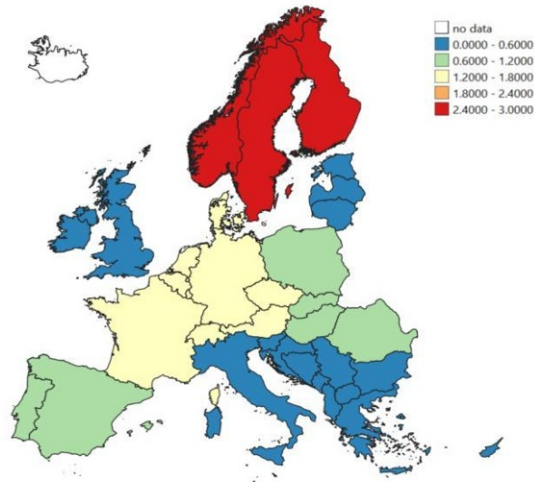
T2+HT2 Exposure (Adult , Medium Bound) - Occurrence data
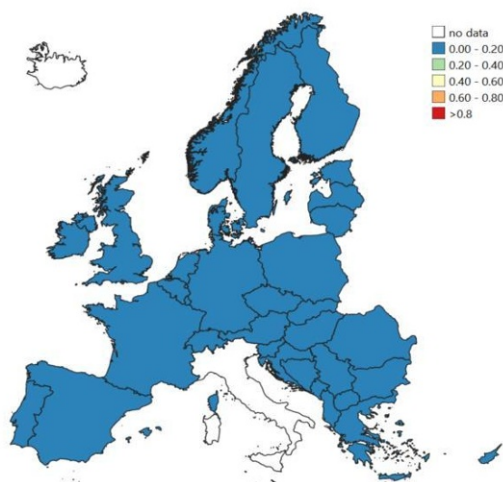
Figure 45: Exposure calculated for T2+HT2 (occurrence dataset) at macro-area level for Adult, MB scenario and mean consumption value.

3.8.2.2. PDI approach

From the MYCHIF biomarker dataset, urine data samples for mixture-1 (DON, FBs, and ZEN) were judged to be quali/quantitatively consistent with the purpose for the PDI approach. A Biomarker dataset was prepared for each mycotoxin including only data sampled in EU countries, merging adolescent and adult groups and choosing healthy people excluding any kind of study where patients were enrolled.

The number of records in the dataset (corresponding to the number of references), number of samples, average and range values for each biomarker of exposure (urine, µg/L) is summarized in Table 37.

Table 37: Number of records, samples, average and range for each biomarker of exposure (urine, µg/L)

Mycotoxin	N° of Records	N° of Samples	Mean values (µg/L)	Min-Max (µg/L)
FBs	3	559	0.028	0.004 - 0.07
ZEN	3	352	0.0267	0.02 - 0.03
DON	528	2090	11.90	0 - 225.2

The selected urine BM value was used for the estimation of single mycotoxin exposure (PDI_{MYCO}) and for the estimation of a PDI for the group of mixtures (PDI_{MIX}).

The formula of reference used for the PDI calculation is derived from the general equation (eq. 4):

$$PDI_{MYCO} \ (\mu g/kg_{bw}) = C_{MYCO} \ x \ V_{URINE} \ x \ 1/kg_{bw} \ x \ 100/ER \qquad (eq. \ 4)$$

where:

C_{MYCO} = mean concentration value for mycotoxin in urine (μg/L)

V_{URINE} =Volume (L) of urine output in 24 h

kg_{bw} = kilogram (kg) of body weight

ER = Excretion Rate (%)

The estimation was also carried out for mycotoxin mixture, PDI_{MIX}, combining the mean concentration value (C_{i_myco}) for each mycotoxin following the equation (eq. 5):

$$PDI_{MIX} \ (\mu g/kg_{bw}) = V_{URINE} \ x \ 1/kg_{bw} \ x \ \Sigma_i \ (C_{i\_myco} \ x \ 100/ER_i) \qquad (eq. \ 5)$$

The HI was calculated for the risk characterization following the equation (eq. 6):

$$HI = \sum_{i=1}^{n} \frac{Exp_i}{RV_i} \qquad (eq. \ 6)$$

where Exp_i: is the exposure of the individual substance in the mixture; RV_i: is the reference value of the individual substance in the mixture (i.e. TDI).

When HI is calculated, a HI ≤ 1 indicates that the combined risk is acceptable, whereas when it exceeds 1, it is considered that a potential concern is possible.

As any other estimation, the PDI_{MYCO} assessment has different uncertainties due to a number of approximations. The approximation on metabolism and toxicokinetics assumption are taken (in fact, the assessment is based considering a fixed excretion % and a fixed urine volumes), while urine dilution issues were totally neglected. Table 38 summarises the sources of uncertainties to be considered in the PDI assessment.

Table 38: Uncertainties on PDI values

Body weight	70 kg is the average value for adults, considering that we combined adolescents and adults, this takes a 13% of variability.
Excretion rate	Values derived from single study or from correlation approximations
Urine volumes	This is an average value of 2 L of fixed urine output. Urine is not corrected for dilution factors.
Data representativeness	Different geographic distribution of data used for PDI model calculation

Each single source of uncertainty for the single mycotoxin is integrated to a combined budget uncertainty to be applied to the PDI calculation of the mixture (PDI_{MIX}). In the case of the mixture, even more variables should be considered for all the unknown toxicokinetics and toxicodynamics combined interferences and for the unknown synergistic/additive possible effects.

The analysis of BM data provided a data-driven insight on the presence of the BM in urine of European population groups (De Santis et. al, in preparation).

4.   Conclusions

The main objective of MYCHIF was to develop an integrated innovative method, supported by modelling, for the risk assessment of mycotoxin mixtures in food and feed, i.e. from fungal growth and mycotoxin production and occurrence to harmonised risk characterisation in humans and animals, promoting complex system understanding and knowledge gaps identification.

Adequate literature was available on cropping systems and mitigation actions at pre- and post-harvest as well as on mycotoxin occurrence in many crops.

In maize, most of the published work related to contamination with *Aspergillus* and *Fusarium* species and the resulting occurrence of AFs, DON, ZEN, FBs, NIV, T-2 and HT-2 toxin. Substantial literature exists on fungal growth and mycotoxin production as well as on factors impacting plant-pathogen interaction. In maize and small grains, including barley, oats, wheat and other grains, preventive actions have been considered worldwide, both at pre-harvest, as part of the cropping system and harvest management, and post-harvest through storage, processing and detoxification. A major role was confirmed in maize for the biological control of *As. flavus* to minimize contamination with AFs.

In rice, a considerable amount of literature exists on *Aspergillus* and *Fusarium* contamination. *Penicillium* species are also able to grow on rice during storage, namely *P. verrucosum*. This fungus has the toxicogenic ability to concurrently produce CIT and OTA; although different studies exist, reports on the mycotoxin contamination of rice are limited compared to those on other cereals.

In grapes, most of the published work relate to the occurrence and the ecology of *Aspergillus* species (predominantly black Aspergilli), to the occurrence of OTA in wines, grape juice, currant and raisin, and to the prevention of OTA production.

The principal nuts of concern include peanuts and some of the most popular tree nuts consumed globally, such as pistachio nuts and almonds. The most frequent mycotoxins detected in nuts are AFs.

As a general finding, a very limited amount of information has been found on mycotoxin co-occurrence in all crops, and it is very rare to find data on the co-occurrence at a single sample level.

Methods of analysis ranged from cheap rapid screening tools, to be used both for lab and field, to more sophisticated confirmatory analytical instruments. The main need regards the availability of screening tools for field-based testing for the detection of more than one mycotoxin or mycotoxin families. Even if high or ultra-performance chromatography systems coupled to mass spectrometry have now been widely reported for their valuable use in the determination of the co-occurrence of multiple mycotoxins in different food commodities. Their remains an urgent need for lower cost, fit for purpose methods characterized by the ability to measure multiple mycotoxins, with lower limits of quantification for all the co-occurring mycotoxins, including their metabolites investigated *in vivo,* as well as *ad hoc* reference materials (matrix-RM and of metabolites), for providing reliable quantitative results. This is required to better determine the toxin mixtures present across many feed and food types and allow the data to better inform stakeholders in terms of their effects on human and animal health. There is also a high potential for tools based on combining spectroscopy with chemometric modelling that can be used to screen crops for mycotoxin contamination to serve as part of decision support tools for actors across the feed and food supply chains.

Regarding the toxicity of multiple mycotoxins mixtures, including toxicokinetic and toxicodynamic, the available literature is rather scarce in terms of number of published papers needed by stakeholders. What is available only covers a very limited combination of mycotoxins. Toxicokinetic data is mainly available for pigs and chickens (as relevant species important from an agro-economic point of view) and rats. Mycotoxin type, dosage, exposure pathway, inter- and intra-species differences have been identified among the most important parameters that may influence the toxicokinetic of mixtures. Concerning the toxicodynamic, the data available could be used to set structure-based models describing the mechanisms of action of certain mycotoxins (e.g. ZEN) in some species (i.e. humans and

pigs). However, they could not support the modelling of TD of mycotoxin mixtures. Concerning the modelling of mixtures' toxicity, some macroscopic endpoints have been modelled on selected species (i.e. pigs and chickens); though, the limited availability of data resulted in a moderate coverage of all the possible combinations of mycotoxin mixtures.

BM studies on mycotoxins have been increasing considerably over the last 10 years, either in toxicological studies and biomonitoring context, both on animal species and humans. As far as the amount and quality of information available, a wide spectrum of observations and indications was available in the published literature. However, there is still a lack of harmonization in the experimental settings and design of biomonitoring studies, in the data collection and in the definition of performance criteria of fit for purpose analytical methods (which are generally taken from the food sector). These issues make extremely difficult to exploit this data for exposure assessment goals. International study guidelines should be prepared to support the production of data, which can better contribute also to toxicological studies.

Biomonitoring approaches for assessing exposure to environmental chemicals should use analytical methods with low limit of detection and quantification and with accurate, robust methodology (e.g. double isotope dilution, selective and specific clean-up steps) capable of measuring low-level concentrations. However, this is frequently not the case and again guidelines would help enormously. It is suggested EFSA convene a working group to address this identified need. Moreover, a further weak point for BM analytics is the lack of analytical standards for the majority of the glucuronide metabolites, the lack of reference material in biological matrix and the lack of inter-laboratory studies, mandatory tool for guaranteeing the comparability of measurements.

The main BM of exposure for the most concerning mycotoxins has been identified, either as parent compounds or altered metabolic forms (e.g. glucuronide, sulphate or hydroxylated ones). It is known that the sampling strategy is a key step but also an important source of variability, adding extra uncertainty to that already related to the toxicokinetic parameters. However, it has been observed that, in most cases, inadequate attention was paid to the sampling strategies (e.g. knowledge on the BM stability, defined time of sampling, way of sample collection and storage).

Regarding BM in animals, the majority of studies has been conducted on AFs followed by DON, OTA, and ZEN, while among the emerging mycotoxins, ENNs has been studied in a small number of publications and other emerging mycotoxins as T2, NIV, BEA and STC have been investigated in only one publication each. The most studied animal species are included in one of the five identified clusters: suidae (pigs and wild boars), bovidae (cow, cattle, buffalo, sheep and goat), poultry (chickens, hens and turkeys), rodents (rat and mice); and other animal species (dog and horses). From the collected information it was found that metabolic patterns change depending on the animal species; therefore, a species-specific information is now available. Urine is the most investigated matrix followed by serum, milk, faeces and bile. Finally, few observations on multi-mycotoxins in any of the possible combination animal species-mycotoxin-matrix were found to be available.

Regarding BM in humans, AFs is the most widely studied mycotoxin followed by OTA, DON, FBs, ZEN and other emerging mycotoxins such as ALT, TEA, FUSX, NEO, CIT, NIV, T2, DAS, and ENNs in a very few studies. Any possible association of metabolic patterns in animals with the human model suffers from a certain degree of uncertainty, depending on the closeness of the animal model to the human. Also, in studies on humans, urine was found to be the most investigated matrix followed by serum/plasma/blood, faeces and breast milk.

Biomarkers represent a good integration of the exposure estimates as they take into consideration the overall exposure to multiple chemicals while providing information on the variability and trends in exposure scenarios. The ability to match the results of BM, which measure the exposure via the internal dose, in the health risk assessment context, which in turn refers to external dietary doses, should count on i) suitable/validated Physiologically-Based Toxico-Kinetics (PBTK) models to properly quantify the biotransformation, metabolism and excretion of all the BM compounds, ii) applicable MOA or adverse

outcome pathway (AOP) and toxicological frameworks to describe biological key events leading to an effect, iii) available flexible approaches to multiple chemicals and iv) a structured schemes of biological fluid sampling and related analysis. Whilst for a number of environmental contaminants a well-structured framework can support the satisfactory interpretation of biomonitoring data in the health risk perspective, also in a context of combined exposure of mixtures, for the mycotoxins issue there is still a lack of important data either for single and combined exposures.

The description of RA scenarios in hierarchy maps was possible for the HCS considering exposure to Mixture-2 (i.e., T2/HT2, DON and NIV) for adults, MB contamination and mean consumption values. Visual hierarchy maps have been used to emphasize risky areas with important exposure values over less important exposure areas in EU countries. The maps were defined for each mycotoxin of the mixture-2 and were possible since occurrence and consumption values were made available in the EU. These maps represent useful pictures where it is immediately noticed where the highest ranks in the visual hierarchy are placed.

5.   Data gaps and recommendations for future work

Pre- and post-harvest management of mycotoxins. Scarce information is available for the co-occurrence of mycotoxins, as well as for the co-occurrence of modified mycotoxins and their fate during processing is not clearly understood. It is therefore suggested to plan research actions to fill such data gaps.

Methods of analysis. Available methods are, in general, satisfactory for the evaluation of the occurrence of mycotoxin mixtures, especially those based on high-resolution mass spectrometry (HRMS). However, the transfer of literature-based methods to the routinely monitoring plans is often difficult, due to the costs of the instrumentation. The development of high-throughput multi-analyte biosensors is promising, but still in the R&D phase. Overall, while perfectly developed for compliance analysis, the routinely methods are not always fit-for-purpose for risk assessment, with inadequate sensitivity, leading thus to an higher percentage of left-censored data in the occurrence database.

Toxicity. Data so far available are overall scarce and the following aspects need to be considered in future investigations to provide a proper ground for modelling:

• The development of prioritisation criteria of mycotoxin mixtures to be tested *in vivo* and *in vitro* is urgently needed since it would be unfeasible to test all possible combinations

• Harmonisation of methodologies and consensus guidelines for generating *in vitro* and *in vivo* TK and TD are needed to provide consistent data for PBTK and BMD modelling of mycotoxin mixtures.

• The texting of possible combinations of mycotoxins mixtures that can really occur in the real world is needed for a broader assessment of mycotoxins

• The use of structure-based TD models (i.e. 3D *in silico* modelling) should be considered to understand the mechanisms of toxicity of mycotoxins, to provide a reasonable foothold to develop prioritization criteria, and to mechanistically explain inter- and intra-species variability

• The use of predictive TK and TD modelling including uncertainty and inter- and intra-species variability analysis should be considered in the future to assist in reducing the overall uncertainty and the production of a more robust risk assessment of chemical mixtures for animals and humans.

Biomarkers. In order to match the information from the quantitative analysis of BM and dietary intakes assessments, and to establish a correlation with the food intake by which animal species and human beings may be reached, the following gaps should be dealt with:

• Need to define models of derive qualitative and quantitative correlation between mycotoxin intake from food and from other possible routes of exposure like contact or inhalation, the latter very important in some working environment like cereal management and store houses;

• Lack of quantitative determination of the metabolic pathway of the parent compounds. There are numerous sources of uncertainties affecting the quantitative determination of the overall metabolic pathway of the parent compounds that makes the PBTK modelling for human a big challenge. Among the uncertainties, i) the low bioavailability rates ascertained with no information on the destiny of the missing percentage not bioavailable *in vivo*, ii) the insufficient toxicological data for defining bioavailability rates for animal species, iii) the insufficient data for toxicokinetic pathway definitions in animals to be transferred in humans (also taking different doses of intake), iv) need of clarification on the critical effects and mode of actions, v) the lack of commercially reference standards of all the metabolised forms found *in vivo* so far, vi) the lack of harmonization in the availability of analytical methods able to detect very low concentration levels of *in vivo* mycotoxins and to search for discovering new metabolic forms in their conjugated forms;

• Complete lack of information on the possible differences in the metabolic pattern of a mycotoxin between its presence *in vivo* as single or as co-occurring with other mycotoxins and of the corresponding toxic effects;

• Overall evaluation of the differences in transferring the scientific knowledge and findings from animal models to humans, in consideration of the high difference of the corresponding biological systems;

• Scarce molecular epidemiology findings derived from the acquisition of the profile of BM;

• The role of the dose of the single toxin on the profile of the metabolic pathways with scarce possibility to correlate the dose itself with food intake and toxic effect as well.

• The use of forward/reverse approaches (i.e. use of the pharmacokinetic knowledge to derive biomonitoring steady state concentrations or adapt steady-state concentrations calculated from the pharmacokinetic model integrating the actual biomonitoring data) to be relied on comprehensive PBTK models. Actually, these models are are scarce and incomplete for mycotoxins. The future availability of PBTK model implementation in addition to dietary dose estimations is a considerable step towards source-to-dose-to-effect prediction of toxicity for mycotoxins.

• Finally, the use of visual hierarchy maps to describe RA scenarios and to emphasize risk areas with important exposure values over less important exposure areas could be a useful tool for risk managers and for communication purposes. The availability of massive occurrence and consumption data in combination with modelling and predicting approaches may optimize the maps representing realistic scenarios.

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Abbreviations

AFalb	aflatoxin-Alb
AFB1alb	aflatoxins B1-Alb
AFB1lys	aflatoxins B1-Lysin
AFB1N7guanine	aflatoxins B1-N7-Guanine
alloTeA	allo-Tenuazonic Acid
AST	aspartate aminotransferase
aw	water activity
BCRP	breast cancer resistance protein
BEN	balkan endemic nephropathy
BM	biomarkers
BM	biomarker
BMD	benchmark doses
BMR	benchmark response
bw	body weight
BWG	body weight gain
CDF plot	cumulative distribution function plot
CFU	colony forming unit
CKD	chronic kidney disease
CL	clearance
DM	dry matter
DMI	dry matter intake
drc	dose-response curves
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELS	extensive literature search
EU	european union
F	bioavailability
FFQ	food frequency questionnaire
FLD	fluorescence detection
FUMzyme	carboxylesterase fumonisin D
GAPs	good agricultural practices
GSPE	grape seed proanthocyanidin extract
HA	humic acid
HFB1	hydrolysed fumonisin B1

HPLC	high performance liquid chromatography
HSCAS	hydrated sodium calcium alimunosilicate
I.p.	intraperitonal
I.v.	intravenous
Kabs	rate of absorption
Ke	rate of elimination
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MixTox	curve fitting and mixture toxicity assessment
MS	mass spectrometry
OHCIT	dihydrocitrinone
OTP	oxidised tea polyphenols
P-gp	P-Glycoprotein
pHFB1	partially Hydrolysed Fumonsin B1
P-P plot	probability–probability plot
Q-Q plot	quantile-quantile plot
RIA	radio-immune assay
Sa	sphinganine
So	sphingosine
T	temperature
TD	toxicodynamics
TK	toxicokinetics
TLC	thin layer chromatography
Tmax	maximum concentration
UV	UV detector
Vd	volume of distribution
VFM	velasco fluorotoxinmeter
y	years
YCW	yeast cell wall

Appendix A – Formats used in WP1
A.1 - Data model for mycotoxin occurrence in crops

Element Name	Element Label	Data Type	Data Type class	Description
DB_origin	Database origin	string	Alphanumeric strings	data provider
sampCountryorigin	Sampling country of origin	string	Alphanumeric character	sample country origin, ISO ALPHA-2 Code
sampContinentorigin	Sampling continent of origin	string	Alphanumeric character	sample continent origin, ISO 7 continents
sampContinent	Sampling continent	string	Alphanumeric character	sample continent trial site/analysis, ISO 7 continents
paramType	Type of parameter	string	Alphanumeric character	toxin parameter
sampCountry	Sampling country	string	Alphanumeric character	sample country trial site/analysis, ISO ALPHA-2 Code
sampRegion	Sampling region	string	Alphanumeric character	sample region trial site/analysis
sampInfo.latitude	Sampling info latitude	floating	Double	sample field trial site, latitude decimal degree N
sampInfo.longitude	Sampling info longitude	floating	Double	sample field trial site, longitude decimal degree N
sampInfo.altitude	Sampling info altitude	floating	Double	sample field trial site, altitude above sea level (m)
sampY	Sampling year	integer	Short	starting year of sampling
sampYearIncreas	Sampling year increase	integer	Short	number of years of sampling
sampSize	Sample size	integer	Short	number of samples
sampMethod	Sampling method	string	Alphanumeric character	sampling methodology
sampPoint	Sampling point	string	Alphanumeric strings	food chain level where the sample has been taken
sampPointInfo	Sampling point information	string	Alphanumeric strings	food chain level sampling point extra info
sampMatType	Sampling matrix type	string	Alphanumeric character	type of sample taken, sub-domain of the matrix catalogue to be used
sampMatbased	Sampling matrix based	string	Alphanumeric character	matrix description (FoodEx2 level)
sampMatInfo	Sampling matrix info	string	Alphanumeric character	extra info for the matrix level description
sampMatCode	Sampling matrix code	string	Alphanumeric character	extra info for the matrix level coding

growingSystem	Growing system	string	Alphanumeric character	matrix production info
anMethRefId	Analytical method identification	string	Alphanumeric character	analytical method identification
resUnit	Result unit	string	Alphanumeric character	measurement unit
resLOD	Result LOD	floating	Double	limit of detection (LOD)
resLODinfo	Result LOD info	string	Alphanumeric character	info limit of detection (LOD)
resLOQ	Result LOQ	floating	Double	limit of quantification (LOQ)
resLOQinfo	Result LOQ info	string	Alphanumeric character	info limit of quantification (LOQ)
exprResPerc	Percentage of moisture	floating	Double	sample moisture (%)
TOTresValUncertSD	Result value uncertainty Standard deviation for meanTot	floating	Double	standard deviation for meanTot sample values
meanTot	Mean of total values	floating	Double	mean of total sample values
POSresValUncertSD	Result value uncertainty Standard deviation for meanPos	floating	Double	standard deviation for meanPos sample values
meanPos	Mean of positive values	floating	Double	mean ot positive sample values
median	Median value	floating	Double	median of sample values
medianInfo	Median value Info	string	Alphanumeric character	info for median of sample values
min	Min value	floating	Double	min of sample values
max	Max value	floating	Double	max of sample values
>LOD (%)		floating	Double	% of samples >LOD
IQRmin	Interquartile min	floating	Double	min of interquartile
IQRmax	Interquartile max	floating	Double	max of interquartile
Concentration		floating	Double	concentration sample value
DataSampLevel	Data at sample level	logic	Boolean	Data at sample level
Co-occurrence	Data on co-occurrence	logic	Boolean	Cooccurrence
Ref	Reference	string	Alphanumeric strings	Paper Reference

Appendix B − Formats used in WP2

B.1 - Standard data model to collect key data of toxicity *in vivo* parameters. Example on data extraction of key toxicity parameters for the 1976-2010 time frame

Indicator/category	Explanation	Example
Refence	Reference paper with the 1st author name and year of pubblication	Grenier et al.
DOI	The Digital Object Identifier (DOI) of the paper	10.1007/BF01139334
Species	Animal species tested in the paper	Broiler chicken
Sex	Sex of the animal	male
Weight	Body weight of the animal at the start of the experiment	409
Weight.sd	Standard deviation	3
Weight.unit	The unit of measurement	g
Number_of_animals	Number of animals used in the experiment	5
Mycotoxin	Mycotoxin that was administrated	DON
Production	Mycotoxin source	Commercial (Sigma Aldrich)
Purity	Mycotoxin purity	>96
Purity.percentage	Percentage of purity	%
Origin	Origin of extracted mycotoxins	*Aspergillus parasiticus*
Dose	The amount of mycotoxin	0.5
Dose.sd	Standar deviation	0.5
Dose.unit	The unit of measurement	mg/Kg
Other compounds	Presence of additional chemicals	2-acetylaminofluorene
Other_compound_concentration	Concentration of other compounds	20
Other_compound_concentration.units	Units of measurement	mg/Kg
Matrix	Source of mycotoxins exposure	feed
Route_of_administration	Route of mycotoxin admistration	Oral
Exposure	Lenght of exposure	7
Exposure.units	Units of mesurements	day

Note: the last part of the template includes the measured endpoint with the respective standard deviation and units of measurement. As an exmaples, "Bodyweight_gain", "Bodyweight_gain.sd" and "Bodyweight_gain.unit" indicate the gain of body weight (e.g. 140), the standard deviation (e.g. 0.94) and the unit of masurements (e.g. g), respectively.

B.2 - Example on data extraction of key toxicity parameters *in vivo* for the 2010-2017 time frame

Indicator/category	Explanation	Example
Author	Reference paper with the 1st author name	Smith
Year	Year of pubblication	2011
Title	Title of paper	The Impact of the Fusarium Mycotoxin Deoxynivalenol on the Health and Performance of Broiler Chickens
Species	Animal species tested in the paper	Broiler chicken
Strains	Strain of animal used	Ross 308
Sex	Sex of the animal	male
Initial age (day)	Initial age of animals tested	1
Initial body weight (g)	Initial body weight of animal tested	41.9
Sample size	Number of animal per sex per experiments	6
Mycotoxin	Mycotoxin that was administrated	DON
Time of exposure (days)	Length of treatment	35
Dose in the feed (mg/kg feed)	The abundance of mycotoxin in the feed	1
Dose (mg/bw)	The amount of mycotoxin used to treat animal expressed as mg on body weight	2

B.3 - Example on data extraction of key toxicokinetic parameters *in vivo*

Indicator/category	Explanation	Example
reference	Reference to the paper with name of first author and year	Han et al 2012
DOI	The digital object identifier of the paper	10.1039/c2ay25891a
Species	Animal species tested in the paper	Broiler chicken
Sex_species	Sex of the animal	male

Region	Where the study took place	UK
s_size	Number of animals	6
Exposure days	Number of days the animals were exposed to the toxin	5
Toxin.1	1st Mycotoxin that was administrated	DON
Dose.1	The amount of the chemical given in a solution	0.5
Units.1	The units of the amount of chemical given	mg/kg bw
Admin.route	Route of administration	oral
Exposure.dose	If the exposure to administrated dose of the toxin was in a single dose or in an amount of days	Single dose
Exposure.info	Information on state of the animal	fastened
Toxin.2	2nd Mycotoxin that was administrated	ZEN
Dose.2	The amount of the chemical given in a solution	0.5
Units.2	The units of the amount of chemical given	mg/kg bw
Admin.route.2	Route of administration	oral
Toxin.3	3rd Mycotoxin that was administrated	OTA
Dose.3	The amount of the chemical given in a solution	0.5
Units.3	The units of the amount of chemical given	mg/kg bw
Admin.route.3	Route of administration	oral
Toxin.4	4th Mycotoxin that was administrated	AFB
Dose.4	The amount of the chemical given in a solution	0.5
Units.4	The units of the amount of chemical given	mg/kg bw
Admin.route.4	Route of administration	oral
AUC	Area under the curve	36.24
AUC_units	Units of AUC	µg/l/h
AUC.SD	The standard deviation of AUC	3.8
Absorption rate	The Lowest Observed Adverse Effect Level value for the chemical	56 µg/kg bw per day
Absorption units	The mean value on the adverse effect observed	/h

Absorption.sd	The units of the Mean observed value	2.7
Bioavailability%	The main statistical descriptor used (Standard Error, Standard Deviation)	92%
Bioavail.SD	The value of the statistical descriptor used	5.6
$T_{1/2}$el.h	Elimination half life	1.36 hours
$T_{1/2}$el.h info	Description on the type of numeric value of the elimination half life (i.e. single value, range)	Single value
$T_{1/2}$el.h sd	Standard deviation of the elimination half life	0.29 h
cmx	The maximum concentration of the toxin observed	15.9
cmx_units	The units of the cmx	ng/ml
Cmx.sd	The standard deviation of cmx	4.5
tmax	The time the maximum toxin concentration was observed (in hours)	15 h
Tmax.sd	The standard deviation of tmax	1.44
vmax	Maximum elimination capacity	6.4
v.sd	Standard deviation of vmax	0.17
Vmx_units	The units of vmx	L/kg
Ke	Constant of elimination	0.53
Ke_units	Units of the elimination constant	/h
SD-SE	Standard deviation or standard error of elimination constant	0.11
Cl	Clearance	0.34
Cl_units	The units of clearance	L/h/kg
Cl.SD	Standard deviation of clearance	0.03
Ex_route	Excretion route of the chemical	urine
Excretion.rate	The rate of excretion of the chemical	0.27%
Ex_timeobs	The time the excretion rate was measured	72h
C_over_rate	The carry over rate of the chemical	15.51%
Targ_tissue1	The 1st target tissue	Kidney
Targ1_toxin1	The 1st toxin for the 1st target tissue	AFB1

Targ1_toxinN	The Nth toxin for the 1st target	AFB2
Targ_tissueN	The Nth target tissue	muscle
TargN_toxin1	The 1st toxin found in the Nth target tissue	AFB1
TargN_toxinN	The Nth toxin found in the Nth target tissue	AFB2

B.4 - Example on data extraction of key toxicokinetic parameters *in vitro*

Indicator/category	Explanation	Example
Reference	Reference to the paper with name of first author and year	Ajandouz et al 2016
DOI	The Digital Object Identifier (DOI) of the paper	10.3390/toxins8080232
Species	Animal species tested in the paper	Broiler chicken
Sex	Sex of the animal	male
Replicates	Number of experimental replicates	3
Mycotoxin	The mycotoxin used to treat	DON
Dose	Dose of treatment	100
Dose.sd	Standard deviation	3
Dose.unit	Unit of measurements	µM
Cell/Tissue	Cell or tissue type used	Caco-2 cell
Organelle	Organelle used treat mycotoxins	Microsomes
Transport from	Transport direction: where mycotoxins come from	Apical monolayer
Transport to	Transport direction: where mycotoxins were directed	Basolateral monolayer
Transport from	Transport direction: where mycotoxins come from	100
Exposure_time	Time of treatment	6
Transport_time.units	Unit of measuments	h
Phase_I_metabolite	Phase I metabolites produced	4-OH-AOH (free)
Phase_II_metabolite	Phase II metabolites produced	ZEA-glucuronide

Further_metabolites	Additional metabolic transformation	N-C18:0-FB1
Amount_of_remaining_mycotoxins	The amount of not transformed mycotoxins	2.03
Amount_of_remaining_mycotoxins.sd	Standard deviation	0.23
Amount_of_remaining_mycotoxins.unit	Unit of measurment	µM
Excretion_of_mycotoxins/metabolites	The amount of excreted mycotoxin/metabolites	2.03
Excretion_of_mycotoxins/metabolites.sd	Standard deviation	0.23
Excretion_of_mycotoxins/metabolites.unit	Unit of measurment	µM

B.5 - Screening template for mycotoxin mixtures

Mycotoxins in mixture	Species/strain/Age	TK or TD study	Dose response (Yes/No)	Endpoints	Reference
DON+ZEN	pigs, 40kg	TK	No	plasma kinetics, matrix residue levels	(Bannert et al., 2017)
FB1+DON+ZEN+AFB1+OTA	4-week-old piglets (Pietrain/Duroc/Large-white	TK	No	absorption	(Gambacorta et al., 2016)
AFB1+OTA	rats, ten-week-old male Fisher 344	TK and TD	No	absorption (tissue, plasma conc), liver, kidney	(Corcuera et al., 2012)
DON+FB1	broiler chickens	TK	No	various TK parameters, bioavailability	(Antonissen et al., 2015)
DON+OTA+AFB1	toddlers, adults	TK	No	bioaccessibility	(Raiola et al., 2012)
AFB1+T2	Male Sprague-Dawley (SD) rats	TK	No	kinetics and tissue distribution	(Han et al., 2012)

OTA+AFB1	White Leghorn breeder hens, 45 weeks	TK	Yes	tissue residues	(Hassan et al., 2012)
DON+ZEN	1 male adult (human)	TK	No	excretion rate	(Warth et al., 2013)
DON+FB1	Male and female Swiss mice (7-8 week old)	TK and TD	No	lipid metabolism, lymphocytes cells death and renal toxicity	(Kouadio et al., 2013)
DON+ZEN	5 week old Kunming mice male and female	TK and TD	No	serum and liver tissue metabolic profiling	(Ji et al., 2017)
DON+ZEN	pre-pubertal gilts	TK and TD	No	metabolic profile, body weight	(Gajecka et al., 2017)
DON+ZEN+Fusaric acid	32 first-parity Yorkshire sows at 91 ± 3 d of pregnancy	TK and TD	No	protein metabolism, synthesis, lactation	(Diaz-Llano et al., 2010)
Aflatoxins+Fumonisins	Friesian female heifers, 18–21 to 42–45 weeks of age	TK and TD	No	metabolic profile, body weight, growth	(Abeni et al., 2014)
DON+FBs	One-day old Ross 308 broiler chickens	TK	No	various TK parameters	(Antonissen et al., 2017)
Fusarium mycotoxins	one-day-old male Hybrid turkey poults	TK	No	plasma conc, elimination	(Devreese et al., 2014b)
AFB1+OTA+FB1+DON	Non lactating Holstein cows	TK	No	bioavailability	(Pantaya et al., 2016)
DON-3G + ZEN-14G	rats	TK	No	tissue residues	(Versilovskis et al., 2012)

3-ADON +					
15-ADON					

B.6 - Screening template for single mycotoxin data

Mycotoxin	Species/strain/Age	TK or TD study or TK/TD	Dose response/Single dose	Endpoints	Reference
AFB1	pregnant mice	TK	No	Absorption and elimination	(Bastaki et al., 2010)
AOH	Male and female NMRI mice	TK and TD	Yes	various TK parameters and genotox	(Schuchardt et al., 2014)
T-2	Swiss albino female mice	TK and TD	No	absorption and hepatotox	(Chaudhary et al., 2015)
DON	male piglets, 21 days of age	TK and TD	No	absorption, metabolcharect,	(Danicke and Doll, 2010)
DON	piglets, mix gender	TK	No	absorption kinetics	(Devreese et al., 2014a)
DON	weaned male castrated piglets	TK and TD	No	excretion, growth rate	(Danicke et al., 2012)
T-2	male 9-week-old pigs (Landrace)	TK	No	kinetics parameters, absorption	(Goossens et al., 2013)
DON	One-week-old broiler chicks	TK	No	absorption, plasma conc	(Yunus et al., 2010)
DON	Three-week-old female broiler chickens	TK	No	plasma conc, TK parameters	(Pralatnet et al., 2015)
DON	21-day-old broiler chickens	TK	No	different TK parameters	(Devreese et al., 2012)
DON	one day-old chicks (LohmannLSL)	TK	No	excretion	(Danicke and Brezina, 2013)
DON	6-week-old broiler chickens male and female	TD	No	nutrient absorption	(Awad et al., 2014)
AFB1	male Wistar rats	TK	No	absorption	(Hernandez-Mendoza et al., 2011)
Emodin	male Sprague-Dawley rats	TK	No	plasma conc, recovery	(Kong et al., 2011)

AFB1	male Sprague–Dawley rats	TK and TD	No		absorption, hepatotox	(Lu et al., 2017)
Emodin	Sprague-Dawley female rats, 8-10 weeks old	TK	No		TK param, bioavailability	(Di et al., 2015)
OTA	15 week old male and female F344 rats	TK	No		TK param, plasma conc	(Vettorazzi et al., 2010)
DON	broiler chickens 3weeks old, male pigs, 11 weeks old	TK	No		TK param, bioavailability	(Broekaert et al., 2015b)
DON	broiler chickens 3weeks old, male pigs, 11 weeks old	TK	No		TK param, bioavailability	(Broekaert et al., 2017b)
NIV	3-week-old-female broilers	TK	No		TK param	(Kongkapan et al., 2016a)
DON, T2, ZEN	3-week-old broiler chickens	TK	No		TK param, bioavailability	(Osselaere et al., 2013)
ENN B1, ENN B	3-week-old broiler chickens	TK	No		TK parameters	(Fraeyman et al., 2016)
T-2	broiler chickens	TK	No		TK param, bioavailability	(Broekaert et al., 2017a)
T-2	5-week-old broiler chickens (kebao-500)	TK	No		TK parameters	(Sun et al., 2015)
ZEN	3-week-old female broilers	TK	No		TK parameters	(Buranatragool et al., 2015)
ZEN	Turkey Hybrid converter poults male/female, broiler chickens (Ross 308) male/female, laying hens (Brown Leghorn) female	TK	No		TK parameters, bioavailability	(Devreese et al., 2015)
DON	Male B6C3F1, 7 weeks old	TK	No		bioavailability	(Tamura et al., 2013)
ENN B1	piglets	TK	No		bioavailability	(Devreese et al., 2014b)
DON	pigs	TK	No		TK parameters	(Paulick et al., 2015)
DON	pigs	TK	No		TK parameters	(Saint-Cyr et al., 2015)
AFB1	pregnant mice	TK	No		Absorption and elimination	(Bastaki et al., 2010)

B.7 - Example on data extraction of key parameters on biomarkers

Indicator/category	Data Type class	Explanation
Reference	Alphanumeric strings	Reference paper with the 1st author name and year
Type of study	Alphanumeric strings	Biomarkers of exposure Biomarkers of effect Toxicological studies with data on biomarkers Toxicokinetic study with data on biomarkers Validation of the analytical method for biomarker analysis
Specie (H/A)	Double	Human/Animal
Single or Multiple Biomarker Study	Double	(M) for multibiomarker or (S) for single biomarker study
ParamType_Mycotoxin	Alphanumeric character	Mycotoxin source of exposure (ex: case of AFB1 toxin, and AFM1 biomarker)
ParamType_Biomarker	Alphanumeric character	Biomarker molecule/molecules measured
Substrate	Alphanumeric character	Indicate the substrate (urine, serum, human milk, etc.) where the biomarker is measured
sampCountry	Alphanumeric character	
SampleSize	Double	Size of the population involved
SampleType	Alphanumeric character	Infants: up to and including 11 months Toddlers: from 12 up to and including 35 months of age Children: from 36 months up to and including 9 years of age Adolescents: from 10 up to and including 17 years of age Adults: from 18 up to and including 64 years of age Elderly: from 65 up to and including 74 years of age Very elderly: from 75 years of age and older
SampleType_INFO_description	Alphanumeric character	Sex info
SampleType_INFO	Alphanumeric character	Other info
Age	Double	Age in years
SampPeriodYear	Alphanumeric character	Period or year of sampling
meanPos	Double	Value
Median	Double	Value
GeometricMean	Double	Value
SD	Double	Value
Percentile	Double	Value
Percentile_ INFO	Alphanumeric character	Which percentile is available
resUnit_Unit	Double	Units which the values are referred to
Min	Double	when< LOD use (-1)
Max	Double	Value

Creatinine/albumine corrected	Double	1 (=Y); 0 (=N)
Biomarker Creatinine/albumine_Value	Double	Value
Biomarker creatinine/albumine_resUnit_Unit	Alphanumeric character	Report the units t which the values are referred to
Biomarker_info	Alphanumeric character	Additional description/information
anMethRefId_Analytical method	Alphanumeric character	Type of analytical technique
Method Validation_Y(1)_N(-1)	Double	1 (=Y); 0 (=N)
Method Validation_Info	Alphanumeric character	i.e. Reference for the validation used
resLOQ_Method LOQ	Double	Value
resLOD_Method LOD	Double	Value
LOQunit	Alphanumeric character	Report the units which the values are referred to
LODunit	Alphanumeric character	Report the units which the values are referred to
Other_parameter_INFO	Alphanumeric character	
Sampling strategy used_INFO	Alphanumeric character	sampling strategies used for sample collection (i.e. urine 24 h)
Correlation_food_Y(1)_N(-1)	Double	If there is correlation with food: 1 (=Y); 0 (=N)
Any correlation with food intake_INFO	Alphanumeric character	Strategies used for correlation
Exposure assessment_Y(1)_N(-	Double	If there is exposure assessment calculations: (=Y); 0 (=N)
Values ExpAss_Value	Double	
Values expass_Unit	Alphanumeric character	
Biomarker validation process_INFO	Alphanumeric character	

B.8 - List of recurrent indicators and explanation retrieved

Indicator	
Mycotoxin Single	71%
Mycotoxin Multi	29
Biomarker	AFB1-Lys AFB1-N7Gua AFM1 AFOH AF-alb FB1 DON DON-3-sulfate

	DON-15-sulfate DON-GlcA DOM1 DOM-1-GlcA ZEN ZEL α+β ZEA14GlcA OTA OTα Allo-tenuazonic acid ALT HO-CIT
Substrate	Plasma (14%) Urine (41%) Breast milk (8%) Blood (4%) Serum (19%) Animal milk (7%) Other (11%)
sampCountry	**ISO TABLE - 2 LETTERS** **(22 countries)** AT3% BD5% BR3% DE5% IT6% KE4% MY2% PT 2% TZ 3% US 7% Others 60%
SampleType	Toddlers: 6% Children: 5% Adolescents: 1% Adults: 57% Elderly: 1% Animals: 30%
SampleType_INFO	Summer_Rural Summer_Urban Winter_Rural Pregnant woman_Rural Pregnant woman_Suburban Pregnant woman_Rural and suburban Workers_Exposed _Non exposed to dusts in two mills Undernutrition and diarrheal disease Incidence of undernutrition and diarrheal disease Recurring aflatoxicosis_Group I placebo Recurring aflatoxicosis_Group I treatment Recurring aflatoxicosis_day 1 Recurring aflatoxicosis_day 21 Women_Mixed diet

	Mothers with no habit of mouldy food consumption
	Healthy German
	Healthy Turkish origin
	3 villages Kigwa, Nyabula and Kikelelwa in Tabora, Iringa and Kilimanjaro regions Visit1
	3 villages Kigwa, Nyabula and Kikelelwa in Tabora, Iringa and Kilimanjaro regions Visit2
	Control
	Celiac Disease
resUnit_Unit **Biomarker** **creatinine/albumine_resUnit_Unit**	pg/mg albumin pg AFB1-lys/mg albumin ng/mg creatinine pg/mg creatinine pg/ml ng/ml ng/l ug/l ug/day ug/kg
Analytical method	UHPC-MS/MS_ESI LC-MS/MS HPLC-MS IDMS HPLC-FC HPLC-FL UPLC-MS ELISA EH + LLE + LC–MS/MS UHPC-MS/MS_ESI UPLC-MS/MS
Type of sampling strategy used	Samples collected at 15 months Good health participants Morning urine (Monday and Friday) First morning void urine Cow's milk and other milk for investigation of human exposure to AFM1 First morning void urine 24 h urine 50 ml of breast milk samples Urine samples were collected at the first visit Blood samples were collected at the first visit 10 volunteers for method verification

Appendix C — Formats used in WP3

C.1- Encoding Keys

Encoding Keys
missing value = -999
value<LOD = -1
value<LOQ = -2
data at sample level (yes=1; not=0)
cooccurrence (yes=1; not=0)

C.2 - Paramtype (toxins coding)

Code	Toxins
15AcDON	15-Acetyl-deoxynivalenol
15OHculmorin	15 OH culmorin
2AOD3ol	2-Amino-14,16-dimethyloctadecan-3-ol
3AcDON	3-Acetyl-deoxynivalenol
3NP	3 nitropropionic acid
5OHculmorin	5 OH culmorin
AcDONs	acetyldeoxynivalenols
AFB1	Aflatoxins B1
AFB2	Aflatoxins B2
AFG1	Aflatoxins G1
AFG2	Aflatoxins G2
AFM1	Aflatoxin M1
AFs	Aflatoxin total
AFalb	Aflatoxin-albumin
AFB1alb	Aflatoxins B1-albumin
AFB1lys	Aflatoxins B1-lysin
AFB1N7guanine	Aflatoxins B1-N7-guanine
ALT	altenuene
Alterperylenol	Alterperylenol
AME	alternariol monomethyl ether
AME3G	alternariol monomethyl ether-3-glucoside
AME3S	alternariol monomethyl ether-3-sulfate
AND A	Andrastin A
Antibiotic Y	Antibiotic Y
AOH	Alternariol
AOH3G	alternariol-3-glucoside
AOH3S	alternariol-3-sulfate
Asterric acid	Asterric acid
ATE	altenuene
ATX1	Altertoxin I
ATX2	Altertoxin II
Aurofusarin	Aurofusarin
Avenacein Y	Avenacein Y

Averufin	Averufin
BEA	Beauvericin
Butenolide	Butenolide
CER	Cereulid
Chanoclavine	Chanoclavine
Chlamydospores	Chlamydospores
CIT	Citrinin
CTN	Citreoviridin
CPA	Cyclopiazonic acid
Culmorin	Culmorin
Curvularin	Curvularin
Cytochalasin E	Cytochalasin E
DAS	diacetoxyscirpenol
DASG	diacetoxyscirpenol
Deepoxy HT2	de-epoxy-HT2
Deepoxy T2	de-epoxy-T2
DHT	dihydrotestosterone
DON	deoxynivalenol
DON3G	deoxynivalenol-3-glucoside
DON3glucuronide	deoxynivalenol-3-glucuronide
DOM1	de-epoxy-deoxynivalenol
DOM1glucuronide	de-epoxy-deoxynivalenol-glucuronide
Emodin	Emodin
ENA	enniatin A
ENA1	enniatin A1
ENB	enniatin B
ENB1	enniatin B1
ENB2	enniatin B2
ENB3	enniatin B3
ENB4	enniatin B4
ENNs	enniatins
Equisetin	Equisetin
Ergocristine	Ergocristine
Ergocomine/-corminine	Ergocomine/-corminine
Ergocornine	Ergocornine
Ergocorninine	Ergocorninine
Ergocristine/-cristinine	Ergocristine/-cristinine
Ergocryptine	Ergocryptine
Ergocryptinine	Ergocryptinine
Ergometrine	Ergometrine
Ergometrine/-metrinine	Ergometrine/-metrinine
Ergometrinine	Ergometrinine
Ergonovine	Ergonovine
Ergoscristine	Ergoscristine
Ergosine	Ergosine
Ergosinine	Ergosinine
Ergotamin	Ergotamin
Ergotamine	Ergotamine
Ergotaminine	Ergotaminine
Ergosterol	Ergosterol

EtOTB	OTB ethyl esters
EAs	Ergot alkaloids
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B3
FB4	Fumonisin B4
FBs	Fumonisin total
Festuclavine	Festuclavine
FUS	Fusaproliferin
FUSX	Fusarenone X
HFBs	hydrolysed fumonisins B
HFB1	hydrolysed fumonisin B1
HT2	HT-2
HT23G	HT-2 toxin-3-diglucoside
HT2G	HT-2 toxin-diglucoside
HT3	HT-3
HT4	HT-4
HT5	HT-5
IsoTEN	IsoTEN
KojicAcid	KojicAcid
LSD	lysergic acid diethylamide
MacrosporinA	MacrosporinA
MalforminC	MalforminC
MARCFORTINE A	MARCFORTINE A
MAS	monoacetoxyscirpenol
MeOTA	methyl Ochratoxin A
MeOTB	methyl Ochratoxin B
MON	moniliformin
MPA	methylphosphonic acid
NEO	neosolaniol
NEOG	neosolaniol-3-glucoside
NIV	nivalenol
NIV3G	nivalenol-3-glucoside
OHLSD	2-oxo-3-hydroxy lysergic acid diethylamide
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
OTs	Ochratoxins
PAC	penicillic acid
PAT	patulin
Penitrem A	Penitrem A
ROQC	Roquefortine C
ROQE	Roquefortine E
STC	Sterigmatocystin
STO	scirpentriol
T2	T-2
T2 tetraol	T-2 tetraol
T2 triol	T-2 triol
T2G	T-2-glucoside
TeA	tenuazonic acid

alloTeA	allo-Tenuazonic acid
TEN	tentoxin
ZAN	zearalanone
ZEN	Zearalenone
ZEA14G	zearalenone-14-glucoside
ZEA16G	zearalenone-16-glucoside
ZEA4G	zearalenone-4-glucoside
ZEA4S	zearalenone-4-sulfate
αErgocryptine/-cryptinine	αErgocryptine/-cryptinine
αZAL	a-zearalanol
αZEL	α-zearalenol
αZEL14G	α-zearalenol-14-glucoside
αZEL4G	α-zearalenol-4-glucoside
βZAL	β-zearalanol
βZEL	β-zearalenol
βZEL14G	β-zearalenol-14-glucoside
βZEL4G	β-zearalenol-4-glucoside

Appendix D − Guidelines for MYCHIF repository

Partners can upload their data to the repository in any format (text files or images or code). IBIMET will manage everything related to the repository, reviewing folder chain and also, if requested, creating metadata.

During repository implementation, a software platform was installed. OwnCloud (http:/owcloud.com) has been chosen because it fits well with the data management requested by the project. OwnCloud is a self-hosted file sync and share server that provides the creation for community based data-centre. With ownCloud, users have a single interface from which they can access, sync and share files on any device, anytime, from anywhere. Users can quickly find and share the files they need whether shared by others or created by themselves. With features like password protection, link expiration, anonymous and full access sharing, files are managed accordingly. It provides access to data through a web interface, sync clients or WebDAV while providing a platform to view, sync and share across devices easily — all under users and administrator control. ownCloud's open architecture is extensible via a simple, but powerful, API for applications and plugins and it works with any kind of storage.

ownCloud has been chosen also because it is open source, defined as priority in the project, and it is not limited by corporate requirements in terms of new features implementation still preserving the original high performance in terms of security and privacy. Further ownCloud has the ability to configure a shared link as read-write, which means users can seamlessly edit the shared files (protected with a password or not) or upload new files to the server without being forced to sign up to another web service that wants their private data. A LAMP web host, supporting and including Apache, PHP5, MySQL and a URL for remote access were configured and ownCloud service was installed on a virtual machine with the following requisites:

- Ubuntu 16.05.2 LTS
- CPU: 1 - 4 core
- RAM: 2Gb
- HDD: 512 GB

The web access is located at the URL https://mychifrep.fi.ibimet.cnr.it/owncloud/ - A 2048 self-signed key assures a strong protection providing a communications protocol for secure communication on Internet. In the last update of MYCHIF Share repository 14 accounts (at least one user for any partner) are authorized to access, share and modify data. One of the CNR-IBIMET staff is instead configured as group administrator managing the disk space, the numbers of users and their sharing space, taking care of software maintenance and update, enabling applications and synchronizing accounts.

Main screen views are shown in **Fig.D.1** and **Fig. D.2**.
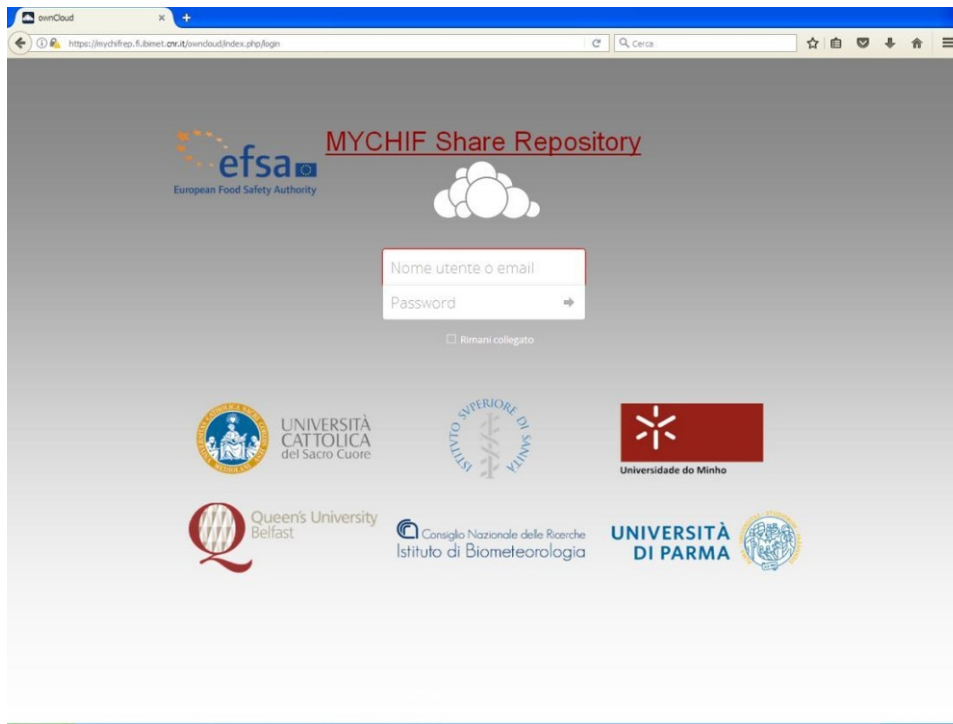
Figure D.1: Login page for MYCHIF repository website
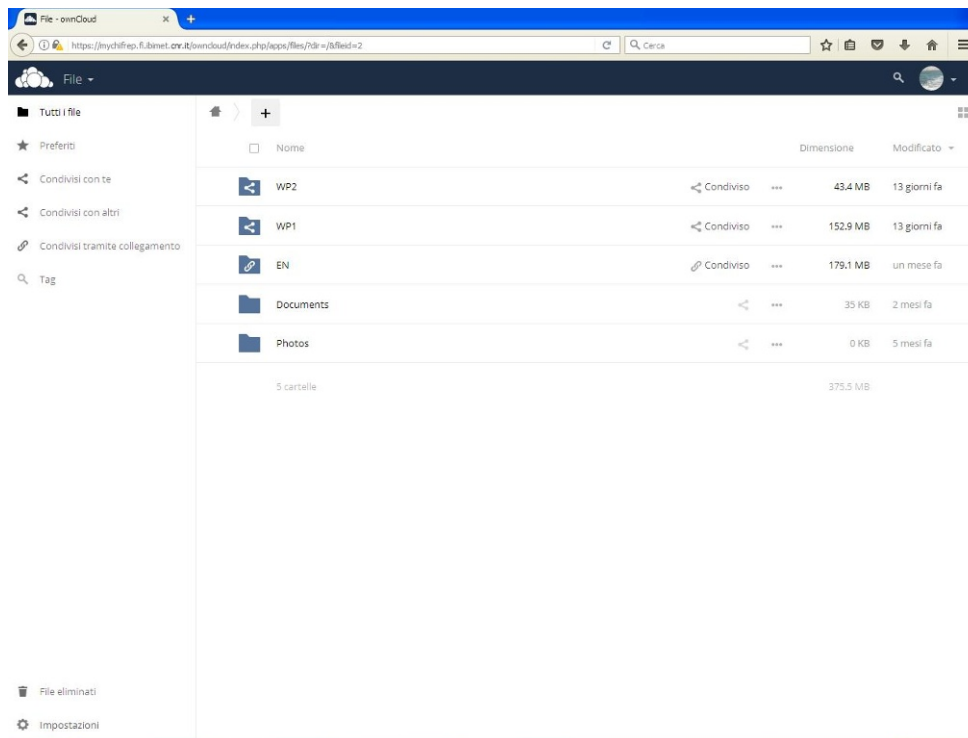
Figure D.2: Sharing files and folders

<u>Repo Maintenance and backup.</u> Once the digital repository is created it needs to be maintained. Administrator provided a self-restore of the whole repository and weekly a data backup of the whole content shared by users.

<u>Practical implications.</u> Guidelines were provided to assist setting up and access to any users. Retrieval of information and knowledge sharing regarding the MYCHIF projects, data extraction and data mining are now possible with the creation of MYCHIF repository.

<u>Literature data mining</u>
The task of targeted data extraction from ELS is heavy and several specific resources for a generic data extraction are widely available on the web. In order to simplify this MYCHIF-workflow a set of tools have been evaluated by using MYCHIF repository with the main aim to extract reliable data from PDF textual documents. Mainly in the software evaluation the skill regarding the data table extraction from the digital documents was taken into account and tested in a group of articles selected by MYCHIF partners.

The evaluation of the tools was carried out taking into account the following features:

1. the licence openness of code
2. the ability to extract data and save them in different format such as .txt .csv and zipped folders
3. the richness of documentation and the availability of an online supporting service
4. the ability to recognize special characters
5. the ability to extract data from tables placed in articles both in vertical and horizontal position

After evaluating their pros and cons we concluded that the most suitable software that could be used in MYCHIF project is Tabula (https://github.com/tabulapdf/tabula). Tabula is an offline software, available under MIT open-source license for Windows, Mac and Linux operating systems, that allows users upload PDF files and extract a selection of rows and columns from any table it may contain. Indeed for MYCHIF project's purposes thank to Tabula it was possible not only a supervised extraction but also a light automation implementing the R tabula-extractor library (https://github.com/ropensci/tabulizer) able to:

1. to read documents stored in MYCHIF repository
2. to perform data extraction
3. to store results in predefined folder in MYCHIF repository

Finally, the MYCHIF workflow of document data extraction could be framed in the following roadmap, where the steps are:

a) Log in to the MYCHIF repository;
b) Upload the files from which you want to extract data in the "Data_pdf" folder (**Fig. D.3**);
c) launch the R extraction code when is possible or perform a supervised extraction (currently available only at CNR-IBIMET);
d) select the section of the table you want to extract, or select all if you are extracting the full table and text. Note: you can always adjust your selection.

e) immediately after making your selection, your data will be immediately stored in the "Data extraction"
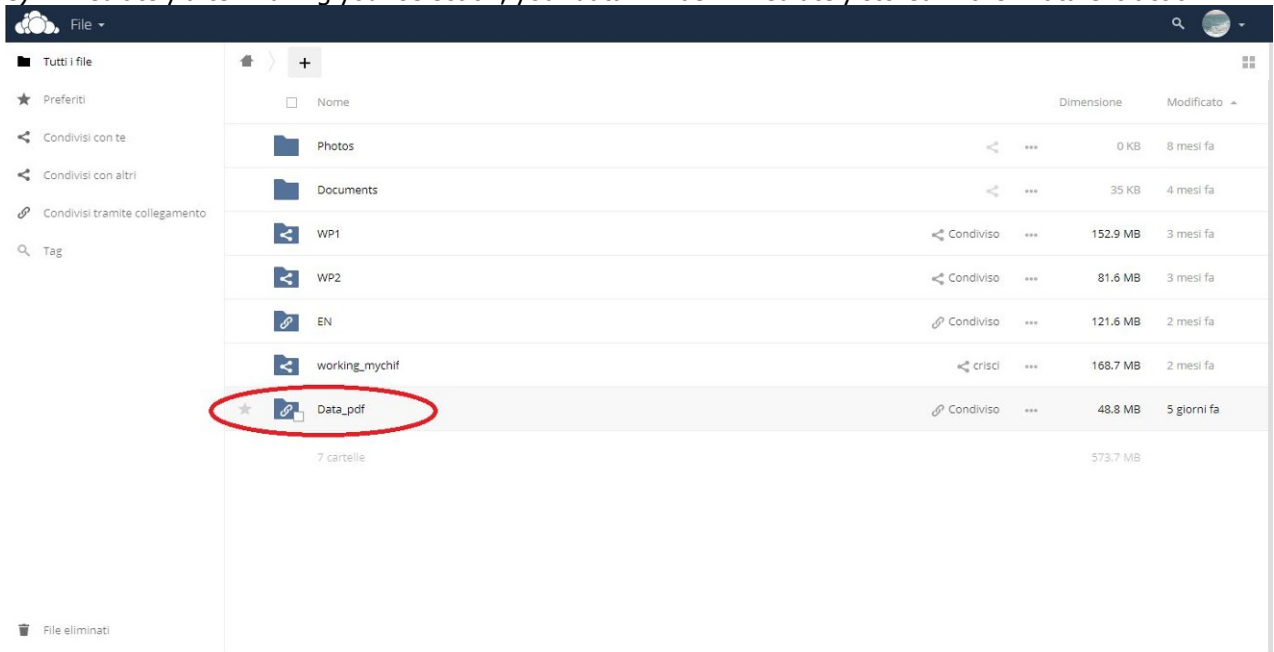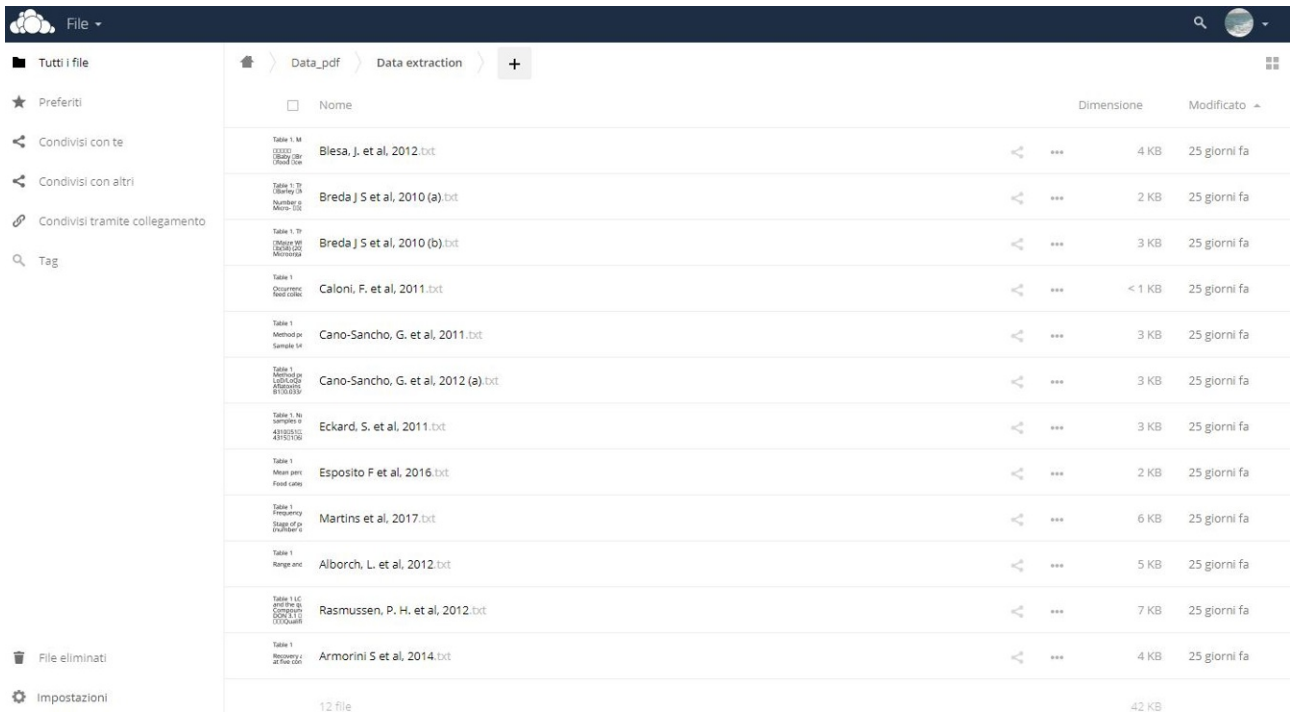

Figure D.3: Uploading PDF filesin the "Data_pdf" folder
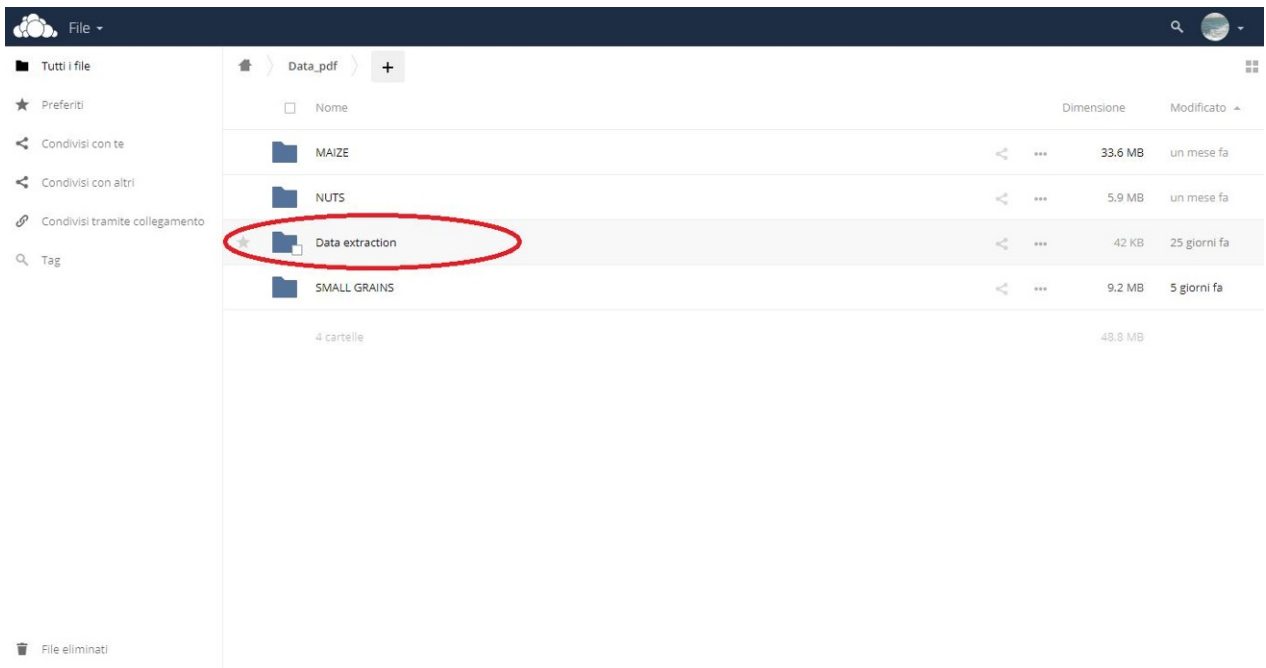
Figure D.4:"Data extraction" folder

The extracted data are saved in txt format (editable with any software) and can be imported into Excel. Each extracted file is saved with the same name as the original pdf file (**Fig. D.5**).

Figure D.5: Extracted .txt files

Furthermore, you have also the option to open the .txt file directly in MYCHIF repository as copy to clipboard and paste wherever you like or as well as you download your txt file which can be opened in any spreadsheet application (Microsoft Excel, LibreOffice Calc, Google Spreadsheet) (**Fig D.6 and D.7**).
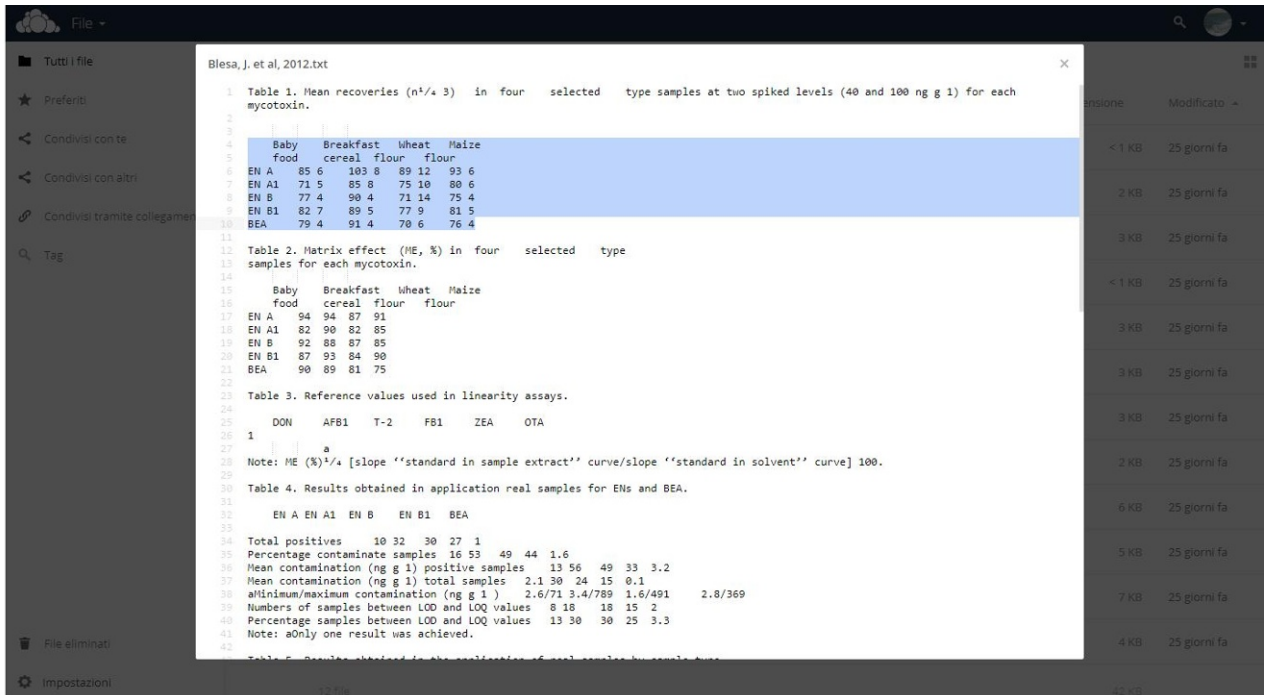
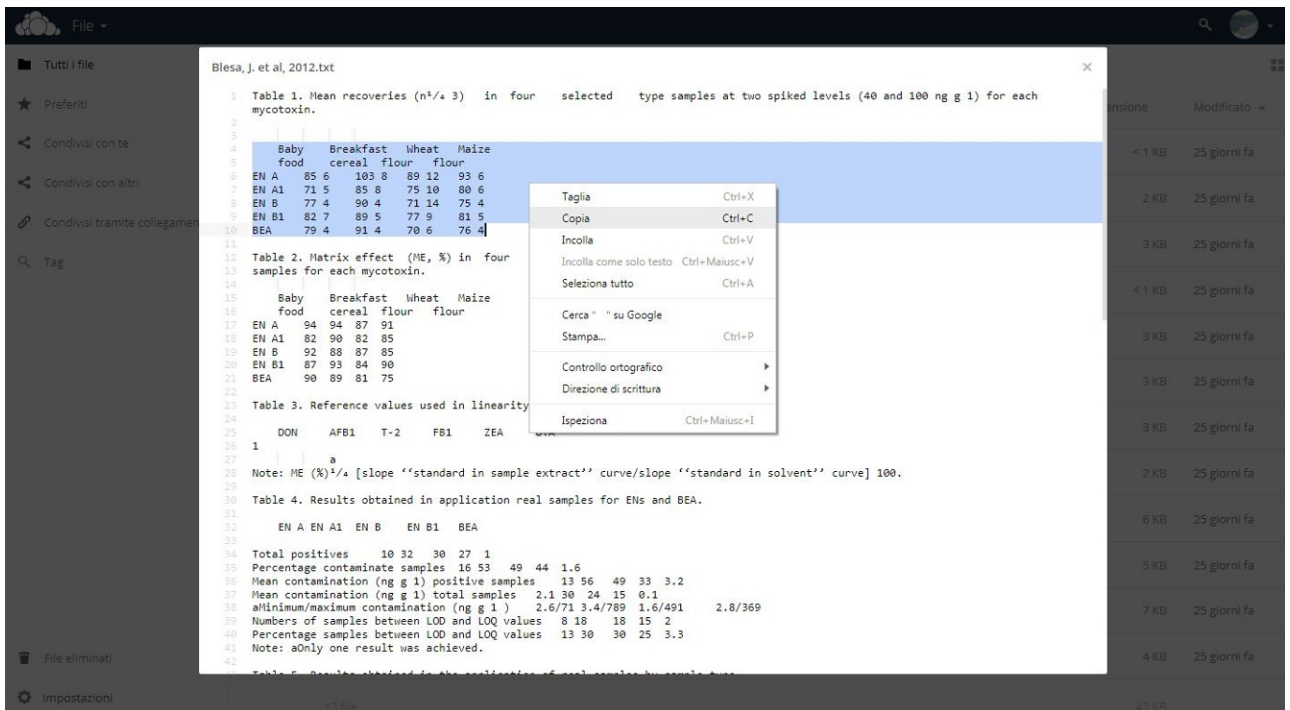**Figure D.6:** Opening .txt files in MYCHIF repository

Figure D.7: Copying .txt files in MYCHIF repository

Annex A − Mycotoxin producing fungi

Annex A can be found online in the MYCHIF platform and EFSA knowledge junction (10.5281/zenodo.3615174).

Annex B − Pre- and post-harvest strategies to mitigate mycotoxin contamination in small grains

Annex B can be found online in the MYCHIF platform and EFSA knowledge junction (10.5281/zenodo.3615174).

Annex C − Pre- and post-harvest strategies to mitigate mycotoxin contamination in nuts

Annex C can be found online in the MYCHIF platform and EFSA knowledge junction (10.5281/zenodo.3615174).

Annex D − Pre- and post-harvest strategies to mitigate mycotoxin contamination in sorghum

Annex D can be found online in the MYCHIF platform and EFSA knowledge junction (10.5281/zenodo.3615174).

Annex E − Pre- and post-harvest strategies to mitigate mycotoxin contamination in spices

Annex E can be found online in the MYCHIF platform and EFSA knowledge junction (10.5281/zenodo.3615174).

Annex F − Methods of analysis for multiple mycotoxin detection, including masked mycotoxins

Annex F can be found online in the MYCHIF platform and EFSA knowledge junction (10.5281/zenodo.3615174).

Annex K − Biomarkers in animals

Annex K can be found online in the MYCHIF platform and EFSA knowledge junction (10.5281/zenodo.3615174).

Annex L − Biomarkers in humans

Annex L can be found online in the MYCHIF platform and EFSA knowledge junction (10.5281/zenodo.3615174).