Aflatoxins in the Soil Environment — Occurrence, Fate and Consequences for the Soil Microbiome and Associated Functions

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Declaration of Authorship

I hereby declare that this PhD thesis, entitled "Aflatoxins in the Soil Environment — Occurrence, Fate and Consequences for the Soil Microbiome and Associated Functions", was conducted and created by my own. All assistances, contributors and authors are declared and clearly indicated in this thesis. This PhD thesis has never been submitted elsewhere for an exam; neither to any other university nor to any other scientific institution.

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Parts of this Thesis, Author Contributions and Third Party Assistance

This cumulative dissertation includes six chapters of which parts have been published as peer-reviewed research papers. In detail, Chapters 2, 4 and 5 are based on the respective accepted manuscripts, retaining their original formatting without any subsequent adjustments made. All research was conducted at the iES Landau, Institute for Environmental Sciences, at the University of Kaiserslautern–Landau (German: Rheinland–Pfälzische Technische Universität Kaiserslautern–Landau, also known as RPTU) in collaboration with the Kenya Agricultural and Livestock Research Organization (KALRO) for field sampling activities in Kenya. Financial support was provided by the "AflaZ: Zero Aflatoxin" project funded by the Federal Ministry of Food and Agriculture (BLE) under the reference AflaZ 2816PROC14. The subsequent section states the use of third party assistance and outlines my and the co-authors' specific contributions to the scientific work.

Third Party assistance

In this thesis, the AI tools ChatGPT (version 3.5), Grammarly and DeepL (Translator) were utilized in the writing process. Throughout the writing process, DeepL was used for translation and Grammarly for grammar and spell checking. The use of ChatGPT was confined to the Introduction (Chapter 1), the Kenyan field study (Chapter 3), and synthesis (Chapter 6) and was not involved in Chapters that were published in peer-reviewed journals, i.e., Chapters 2, 4, and 5. Its practical applications included serving as a tool for improving the overall quality of the text. Specifically, ChatGPT was utilized for tasks such as providing suggestions for phrasing and sentence structure, finding synonyms, acting as grammar and

spell-checker, and aiding in the concise summarization of content. ChatGPT supported the programming and formatting of LaTeX for the preparation of the thesis draft, especially for table formatting and the generation of BibTeX entries for the bibliography.

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Publications

The following chapters of this thesis have been published in peer-reviewed journals:

Chapter 2 Albert, J., More, C. A., Dahlke, N. R. P., Steinmetz, Z., Schaumann, G. E., and Muñoz, K. (2021). "Validation of a Simple and Reliable Method for the Determination of Aflatoxins in Soil and Food Matrices". *ACS Omega* 6.29, pp. 18684–18693. DOI: 10.1021/acsomega.1c01451.

Author contributions: J. Albert and K. Muñoz contributed equally to conceptualizing the study and designing the experiments. The experiments were conducted by J. Albert, C. A. More, and N. R. P. Dahlke. J. Albert analyzed and visualized the data. J. Albert took the lead in writing and finalizing the manuscript. Project supervision was carried out by K. Muñoz and G. E. Schaumann. K. Muñoz was responsible for project administration and funding. The manuscript was finalized through the contributions of all authors.

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Author contributions: J.A. designed the study, conceived the experiments, conducted data analysis, wrote and revised the manuscript; K.M. was involved in the study design, acquired funding, supervised the project and revised the manuscript.

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In addition to the publications listed above that represent Chapters of my thesis, I contributed to the following publications:

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Author contributions: Conceptualization was jointly undertaken by K. G. J. Kenngott, J. Albert, and K. Muñoz. Experiments were carried out by K. G. J. Kenngott and J. Albert. Data analysis and visualization was carried out by K. G. J. Kenngott. K. G. J. Kenngott took lead in writing and finalizing the manuscript. Project supervision was carried out by K. Muñoz and G. E. Schaumann. K. Muñoz was responsible for project administration and funding.

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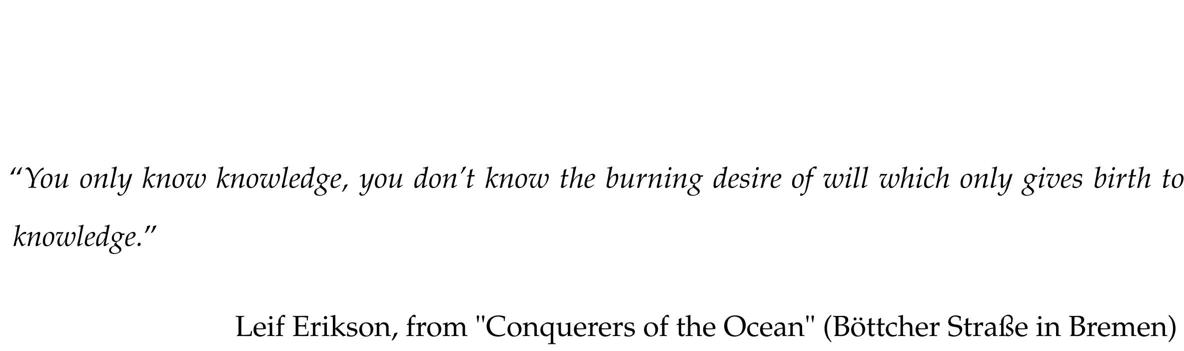
Author contributions: Z. Steinmetz took the lead in setting up the package and programming and describing most of the functions. J. Albert provided code and description for the "matrix_effect" function. J. Albert and K. G. J. Kenngott contributed to the coding and description of the "weight_select" function. Z. Steinmetz was responsible for the maintenance of the package.



"You only know knowledge, you don't know the burning desire of will which only gives birth to knowledge."

Leif Erikson, from "Conquerers of the Ocean" (Böttcher Straße in Bremen)

Abstract

Aflatoxins, a group of mycotoxins produced by various mold species within the genus *Aspergillus*, have been extensively investigated for their potential to contaminate food and feed, rendering them unfit for consumption. Nevertheless, the role of aflatoxins as environmental contaminants in soil, which represents their natural habitat, remains a relatively unexplored area in aflatoxin research. This knowledge gap can be attributed, in part, to the methodological challenges associated with detecting aflatoxins in soil. The main objective of this PhD project was to develop and validate an analytical method that allows monitoring of aflatoxins in soil, and scrutinize the mechanisms and extent of occurrence of aflatoxins in soil, the processes governing their dissipation, and their impact on the soil microbiome and associated soil functions. By utilizing an efficient extraction solvent mixture comprising acetonitrile and water, coupled with an ultrasonication step, recoveries of 78% to 92% were achieved, enabling reliable determination of trace levels in soil ranging from 0.5 to 20 µg kg^{-1}. However, in a field trial conducted in a high-risk model region for aflatoxin contamination in Sub-Saharan Africa, no aflatoxins were detected using this procedure, underscoring the complexities of field monitoring. These challenges encompassed rapid degradation, spatial heterogeneity, and seasonal fluctuations in aflatoxin occurrence. Degradation experiments revealed the importance of microbial and photochemical processes in the dissipation of aflatoxins in soil with half-lives of 20 - 65 days. The rate of dissipation was found to be influenced by soil properties, most notably soil texture and the initial concentration of aflatoxins in the soil. An exposure study provided evidence that aflatoxins do not pose a substantial threat to the soil microbiome, encompassing microbial biomass, activity, and catabolic functionality. This was particularly evident in clayey soils, where the toxicity of aflatoxins diminished

significantly due to their strong binding to clay minerals. However, several critical questions remain unanswered, emphasizing the necessity for further research to attain a more comprehensive understanding of the ecological importance of aflatoxins. Future research should prioritize the challenges associated with field monitoring of aflatoxins, elucidate the mechanisms responsible for the dissipation of aflatoxins in soil during microbial and photochemical degradation, and investigate the ecological consequences of aflatoxins in regions heavily affected by aflatoxins, taking into account the interactions between aflatoxins and environmental and anthropogenic stressors. Addressing these questions contributes to a comprehensive understanding of the environmental impact of aflatoxins in soil, ultimately contributing to more effective strategies for aflatoxin management in agriculture.

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Chapter 1

Introduction

1.1 Aflatoxins in the Food Production Chain

1.1.1 Aflatoxins: The Hidden Danger in Foods

Mycotoxins, produced as secondary metabolites by certain anamorphic fungal species, are natural contaminants in various food commodities, capable of causing disease and death in humans and animals upon consumption (Bennett and Klich, 2003). It is assumed that about 60 - 80% of the global food crops are contaminated with mycotoxins (Eskola et al., 2019). Roughly, more than a thousand mold species have been identified, of which more than 500 produce mycotoxins that have been classified as potentially toxic to vertebrates (Haque et al., 2020). Major mycotoxins or groups of mycotoxins occurring in food that affect human and animal health include aflatoxins, ochratoxins, trichothecenes, fumonisins, alternariol, patulin and citrinin and *Alternaria* toxins. These fungal toxins are produced by species within the genera *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*.

Aflatoxins (AFs), a group of mycotoxins that are produced by several species of the genus *Aspergillus*, are widely considered the most relevant mycotoxins from a food safety point of view due to their widespread occurrence and high toxicity to humans (Afsah-Hejri et al., 2013; Jallow et al., 2021). To date, more than 20 aflatoxin molecules have been identified, with aflatoxin B1 (AFB1) exhibiting the highest toxicity to animals and humans (Ismail et al., 2018). In addition to aflatoxin B1, the aflatoxins B2 (AFB2), G1 (AFG1), and G2 (AFG2), as well as the derivative M1, which is primarily found in milk, are also of toxicological concern (Caceres et al., 2020; Haque et al., 2020). All aflatoxins are derivatives of dihydrofurancoumarins and share a common polycyclic structure derived from a coumarin nucleus linked to a bifurano system (Abrehame et al., 2023; Nazhand et al., 2020). They can be categorized into two chemical groups based on the binding to the dihydrofurancumarin structure: the difurocoumarocyclopentenone series (e.g., AFB1, AFB2, AFB2A, AFM1), characterized by the linkage to a pentanone ring, and the difurocoumarolactone series (e.g., AFG1, AFG2), characterized by the linkage to a lactone ring (Figure 1.1, Abrehame et al., 2023; Nazhand et al., 2020). In addition, AFs can be further divided into AFs with (AFB1, AFB2, AFM1) and without (AFB2, AFG2, AFB2a) a double bond in the 8,9-position in the bifurano system (Abrehame et al., 2023).

Figure 1.1: Structures of major important aflatoxins: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin B2a (AFB2a), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and aflatoxin M1 (AFM1).

1.1.2 Aflatoxin Occurrence in the Food Production Chain: Global Distribution and Geographical Variations

Aflatoxin contamination is prevalent in various crops and regions worldwide, however, certain crops and conditions are more susceptible to AF contamination. In general, oil- and starch-rich crops grown in (sub)tropical regions are the most susceptible to *Aspergillus* infestation and aflatoxin contamination (Rushing and Selim, 2019; Jallow et al., 2021). Commodities that are frequently affected by aflatoxigenic fungi include cereals (wheat, sorghum, rice, acha, millet, maize), tree nuts (almond, pistachio, coconut, walnut), oilseeds (peanut, sunflower, cotton seeds, soybean, and sesame) and spices (garlic, black pepper, coriander, turmeric, ginger, and chili peppers) (Awuchi et al., 2022). The susceptibility of crops to fungal infestation and subsequent AF production is influenced by a combination of environmental factors, crop-specific characteristics and the stage of food production chain and can already occur "pre-harvest" in the field or "post-harvest" during storage, transport and processing (Figure 1.2, Jallow et al., 2021).

Fungal growth and aflatoxin production are determined by chemical (pH, oxygen, carbon dioxide, nutrient substrate composition, pesticides), physical (temperature, moisture,

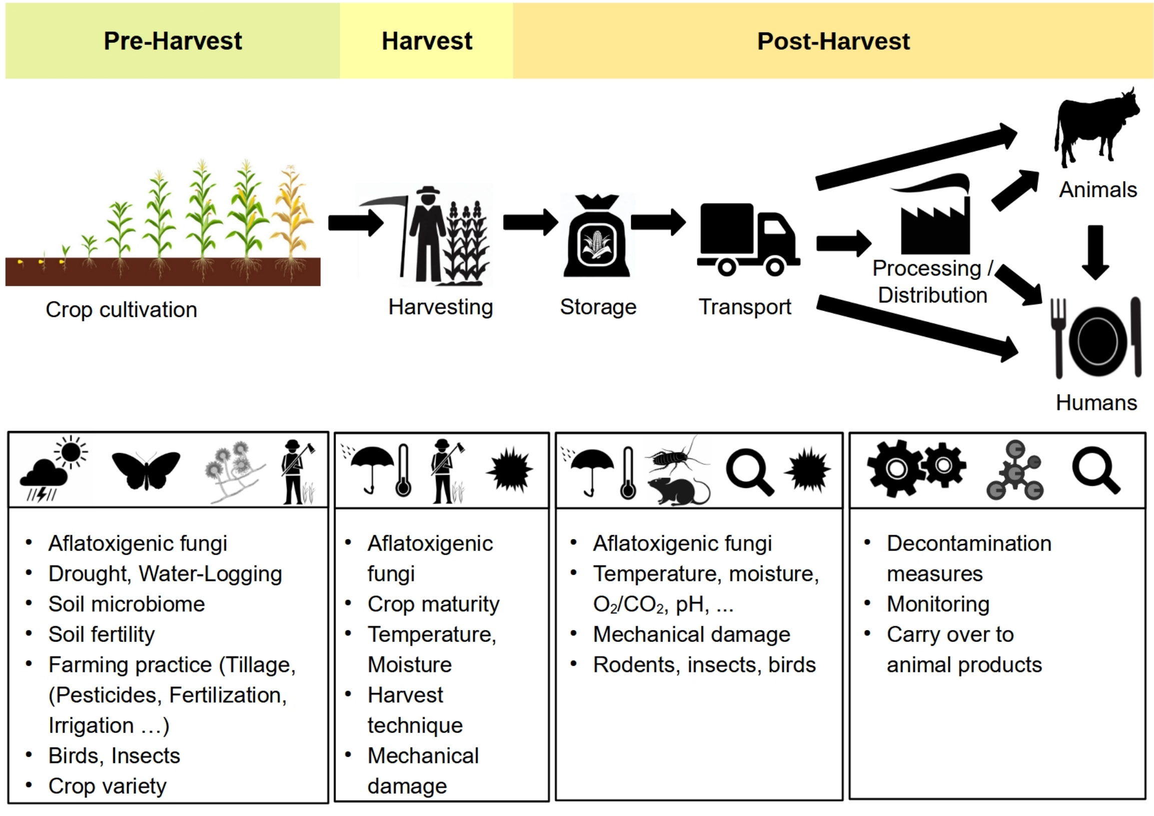

Figure 1.2: Factors influencing aflatoxin occurrence throughout the food and feed production chain from pre-harvest to post-harvest stages.

water activity, mechanical damage) and biological factors (plant variety, seed quality, plant physiology, stress, pest insects, presence of compatible toxigenic fungi, soil microbiome) (Pleadin et al., 2019; Bryden, 2012). In the field, these conditions are primarily determined by weather and site conditions, as well as agricultural practices such as weeding, fertilization, pest control, irrigation, tillage, and harvesting techniques (Figure 1.2). Conditions favoring pre-harvest contamination of crops such as peanuts and maize are high temperatures, insect damage and prolonged drought conditions (Bryden, 2012). During and post-harvest stage, proper crop handling and processing, including adequate drying, clean and dry storage, and protection from rodents and insects, has a significant impact on aflatoxin fungal formation conditions (Figure 1.2, Kyei et al., 2021).

Developing countries from the (sub)tropics are particularly confronted with aflatoxin food contamination, due to the favorable (sub)tropical conditions for the growth and aflatoxin formation of these fungi and the limited access to control measures (Gbashi et al., 2019; Nji et al., 2022a). In a study by Gruber-Dorninger et al. (2019), about 75,000 feed samples collected

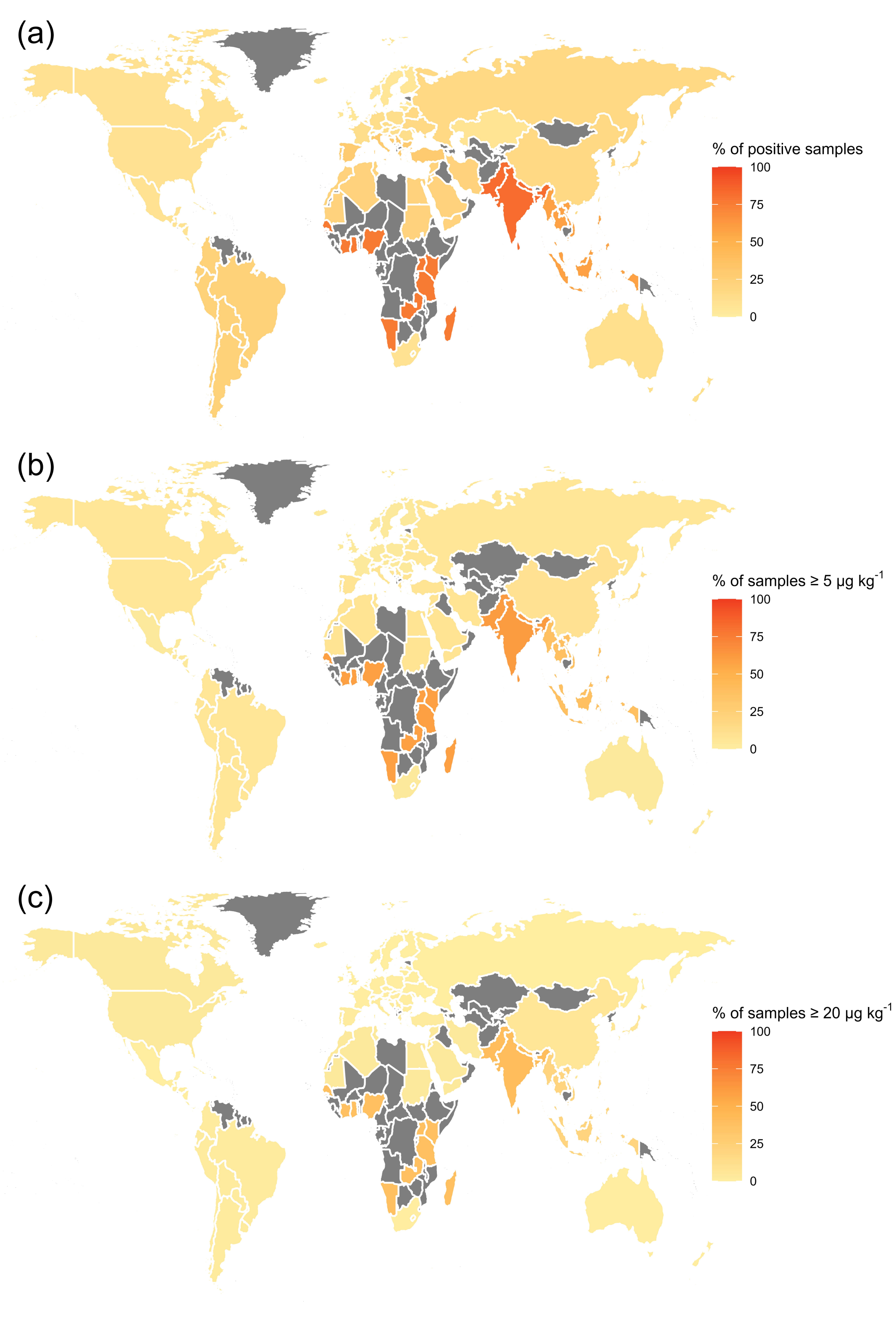

Figure 1.3: World maps showing the prevalence of AFs (sum of AFB1, AFB2, AFG1 and AFG2) in feed samples: (a) Percentage of positive samples; (b) Number of samples exceeding 5 µg kg^{-1}; (c) Number of samples exceeding 20 µg kg^{-1}. Areas filled in grey indicate data not available. The data used for plotting is sourced from Gruber-Dorninger et al. (2019).

from 100 countries from 2008 to 2017 were analyzed for mycotoxins, including aflatoxins. The results showed a clear tendency towards a higher AF prevalence in Southeast Asia (57.4 %), South Asia (82.2 %) and Sub-Saharan Africa (76 %) and a much lower prevalence in Northern (5.9 %) and Central Europe (12.7 %) (Figure 1.3). Likewise, the the number of samples exceeding 5 and 20 µg kg^{-1} AFs are much higher in Southeast Asia (37.9 and 20.9 %), South Asia (61.1 and 41.1 %) and Sub-Saharan Africa (59.1 and 38.5 %), than in Northern (2.4 and 0.4 %) and Central Europe (2.6 and 1.0 %), respectively (Figure 1.3).

These favorable (sub)tropical conditions for aflatoxigenic fungi result not only in higher prevalence but also in significantly higher aflatoxin levels in agricultural products. Table 8.1 (Chapter 8.1) shows the reported aflatoxin contamination levels for various food commodities from countries in different geographical regions, adapted from the compilations by Jallow et al. (2021) and Ismail et al. (2018). Maize and peanuts, major staple foods grown in (sub)tropical regions, are often associated with particularly high levels of aflatoxins (Ismail et al., 2018). For example ΣAFs levels in the range of 10^{3} µg kg^{-1} were reported for cereals and peanuts in Uganda (Sserumaga et al., 2020), Congo (Kamika et al., 2016), Ethiopia (Mohammed et al., 2016), Nigeria (Oyedele et al., 2017), Kenya (Sirma et al., 2016), China (Wu et al., 2016) and Tunisia (Houissa et al., 2019). Spices can also exhibit high levels of contamination, reaching concentrations in this range, as observed in Lebanon (El Darra et al., 2019). In rare cases, ΣAFs concentrations above 10000 µg kg^{-1} can be detected, for example in infant preparations from Mexico (Chala et al., 2013). Furthermore, due to the importance of maize as animal feed - about 55% of maize production is used for this purpose - there is a strong link to the presence of aflatoxins in dairy products (Tolosa et al., 2021). For instance, AFM1 concentrations of up to 4.5 µg L^{-1} have been detected in milk samples from Kenya (Kuboka et al., 2019). These significantly elevated contamination levels mentioned above have profound impacts on both human health and international trade.

1.1.3 Consequences of Aflatoxins: From Human Exposure to Global Trade

The aflatoxin contamination level in foods and feeds, as well as the rate of consumption of the local population, largely determine the extent of human exposure to aflatoxins and the associated health effects (Jallow et al., 2021). The average intake of aflatoxins by humans is estimated to be 10 to 200 ng kg^{-1} day^{-1}, with a total range of 0 to 30,000 ng kg^{-1} day^{-1}

(Kaplan et al., 2003; Gong et al., 2016). Given the high production and consumption rates of staple crops such as maize and peanuts, coupled with a high susceptibility to aflatoxin contamination, these crops are the primary source through which humans are exposed to aflatoxins. It is therefore no coincidence that the highest aflatoxin exposure is consistently reported from developing countries in (sub)tropical regions (Jallow et al., 2021). In this regard, approximately 4.5 billion people in the developing world are at risk of chronic, uncontrolled exposure to AFs (Williams et al., 2004; Rushing and Selim, 2019; Shephard, 2003; Williams et al., 2004). Particular high exposure levels are reported for Sub-Saharan Africa and South-East Asia (Gong et al., 2016) such as Kenya (3.5-14.8 ng kg^{-1} day^{-1}), Swaziland (11.4-158.6 ng kg^{-1} day^{-1}), Mozambique (38.6-183.7 ng kg^{-1} day^{-1}), South Africa (16.5 ng kg^{-1} day^{-1}), Gambia (4-115 ng kg^{-1} day^{-1}), China (11.7-2027 ng kg^{-1} day^{-1}), and Thailand (6.5-53 ng kg^{-1} day^{-1}) (Williams et al., 2004). Meanwhile, exposure levels in developed countries are much lower e.g. 2.7 ng kg^{-1} day^{-1} in the USA (Williams et al., 2004) and 0.93–2.45 ng kg^{-1} day^{-1} in Europe (JECFA, 2008).

Elevated exposure levels are of particular concern because AFs are considered one of the most potent mutagenic and carcinogenic substances known to date (Eskola et al., 2019). The International Agency for Research on Cancer classifies AFB1 and natural aflatoxin mixtures of B, G, and M aflatoxins as group 1 carcinogens (IARC, 2006). Aflatoxins exhibit a toxicity profile where AFB1 is the most potent, followed by AFM1, AFG1, AFB2, and AFG2, with demonstrated acute toxicological and chronic hepatocarcinogenic effects in the liver due to their reactivity with DNA, RNA, enzymes, and proteins (Haque et al., 2020). Chronic aflatoxicosis has been linked to hepatocellular carcinoma or liver cancer, suppresses growth, modulates the immune system, and leads to malnutrition (Rushing and Selim, 2019; IARC, 2006; Haque et al., 2020). In addition, aflatoxicosis can exhibit potent synergistic effects with other factors contributing to liver cancers, such as malnutrition and infection with hepatitis B and C viruses, diseases that are highly prevalent in developing countries – conditions highly prevalent in developing countries, with hepatitis incidences reaching about 20% (Williams et al., 2004). Meanwhile, acute aflatoxicosis has resulted in symptoms such as abdominal pain, vomiting and edema (Eskola et al., 2019). Especially in developing countries in (sub)tropical regions such as Kenya, China, India and Malaysia with favorable conditions for the growth and aflatoxin production of these fungi and limited access to control measures such as safe food storage, severe outbreaks of AFB1 contamination in food frequently occur, resulting in

hundreds of deaths from acute aflatoxicosis (Azziz-Baumgartner et al., 2005; Eskola et al., 2019; Haque et al., 2020).

The impact of aflatoxin contamination in agriculture extends beyond public health, affecting trade and economics in both developed and developing countries (Jallow et al., 2021). In this regard, maize farmers in the US lose $160 million annually due to AFs contamination (Wu, 2015), but losses are higher in developing countries, especially in Sub-Saharan Africa, where they reach losses of $450 million, representing 38% of global agricultural losses due to aflatoxin (Gbashi et al., 2019). Aflatoxins also lead to a significant decrease in agricultural trade between developed and developing countries (Wu, 2015), in part due to the discrepancy between these countries in terms of regulatory limits on the one hand and the prevalence of AFs on the other. In general, AF regulations in non-tropical and industrialized countries, that are less affected by AF problems, are much stricter than in tropical and developing countries, that are heavily affected (Sirma et al., 2018). This discrepancy creates several problems for tropical countries facing the AF problem, such as Sub-Saharan Africa. In these countries, economies are predominantly based on the commercialization of agricultural products (Matumba et al., 2015). The economic importance of agricultural production, combined with a high susceptibility to AFs contamination, has a significant impact on the trade and economy of developing countries in the tropics mainly by reducing the value of the commodities offered for sale (Jallow et al., 2021), e.g. by lowering prices, inspection fees, disposal fees, rejecting or treating lots at extra cost, compensating for claims, and the cost of sampling and analysis in the supply chain (Gbashi et al., 2019). Typically, the main staple foods in these countries are also their most important cash crops (Nji et al., 2022a). As a result, the best-quality crops with the least contamination that meet the standards of non-tropical importing countries are usually exported, leaving the poorer quality, more contaminated crops for local consumption or sale in the informal sector (Matumba et al., 2015; Nji et al., 2022a). This increases the likelihood of the local population consuming AF contaminated foods (Udomkun et al., 2017; Nji et al., 2022a), which result in further economic costs i.e. cost of illness (Meijer et al., 2021).

Contaminated crops unsuitable for sale in the informal food sector or for in-house consumption are often spread in the field for surface decomposition or buried in the soil as organic fertilizer (Fouché et al., 2020). However, the fate and consequences of AFs in soil and on soil organisms that provide important ecological services remain unclear (Fouché et al.,

2020). Consequently, AF contamination could go beyond health and trade issues, potentially affecting soil health, agricultural productivity and food safety.

1.1.4 Regulation of Aflatoxins in Foods and Feeds: Efforts at Local and Global Scales

In order to protect humans and animals from the serious health effects of AFs, most countries have implemented strict regulations (Van Egmond and Jonker, 2004). Regulatory limits are established on sound risk assessments on the basis of toxicological and exposure data, as well as knowledge of the distribution of AF concentrations within potentially susceptible commodities (Chilaka et al., 2022). However, economic and political factors such as trade interests and adequate food supply also have an impact on the setting of local regulatory limits (Van Egmond and Jonker, 2004). In general, the maximum permissible values for AFs in foodstuffs are in the lower µg kg^{-1} range (Van Egmond and Jonker, 2004; Sirma et al., 2018). In most countries, legal limits for the sum of the four major aflatoxins (AFB1, AFB2, AFG1 and AFG2), as well as the most toxic aflatoxin (AFB1), have been set at least for maize and peanuts due to their susceptibility and, at the same time, their importance as staple foods (Wu et al., 2013). A global survey conducted twenty years ago on behalf of the Food and Agriculture Organization of the United Nations (FAO) found that about 100 countries have specific regulations for AFs in various dairy products and feed items (Van Egmond and Jonker, 2004). The legal requirements differed strongly among the countries and regions (Figure 1.4). While for Northern America, Southern America and Europe (inc. Russia) most countries implemented limits for AFB1 and/or the sum of AFs, there is a lack of regulation in Asian and African countries. Further, some free trade zones such as the European Union (EU), Common Market of the South (Mercosur) and Australia/New Zealand harmonized their limits. Although many countries do not have national or international legally binding limits for aflatoxins, it is important to know that many of these countries are members of the Codex Alimentarius Commission (CAC), which sets international standards, guidelines and codes of conduct to facilitate global trade and protect consumers. The CAC has established a standard for aflatoxins in peanuts (sum of AFs $\leq$ 15 µg kg^{-1}), which, while not legally binding at the national level, can be considered a proxy regulation limit (CAC, 1995). A literature search on the situation of AF regulation in 2022 (Annex 8.1, Table 8.2)

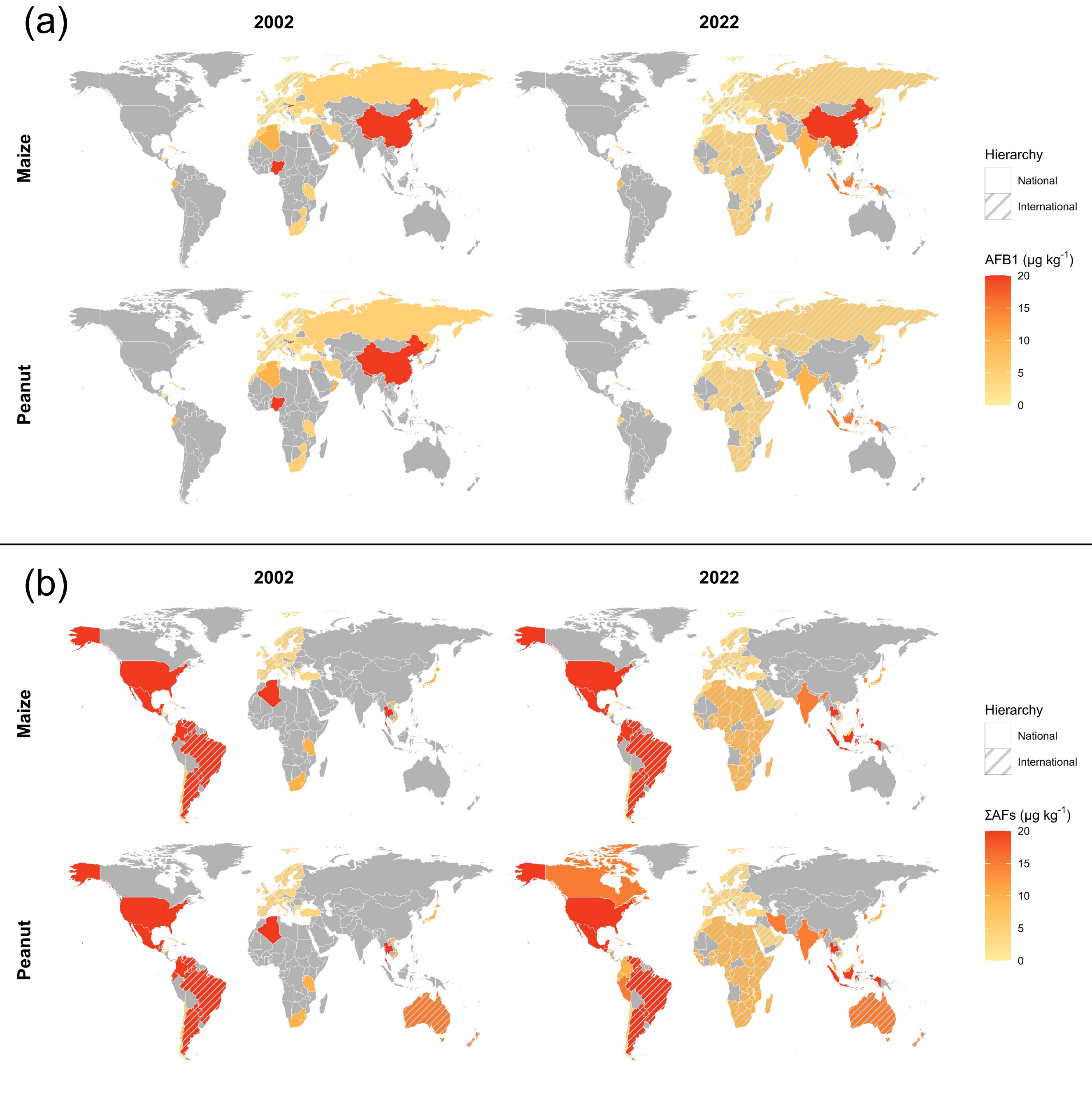

Figure 1.4: World maps showing the regulation limits for AFB1 (a) and the sum of AFB1, AFB2, AFG1 and AFG2 (b) in maize and peanuts in the years 2002 and 2022. The polygons filled with a stripe pattern represent countries that have set limits for aflatoxin through international harmonized standards and those without a stripe pattern represent countries that have set their own national limits.

revealed that an increasing number of countries have introduced limits either at national or internationally harmonized levels and that there is a general trend towards tightening AF limits, especially in African and Asian countries. By joining the European Union, several countries have adopted its particularly strict limits. Regionally harmonized aflatoxin limits have been introduced as a result of the formation of the Eurasian Customs Union (EACU), the East African Community (EAC), the African Organisation for Standardisation (ARSO) and the Gulf Cooperation Council Standardization Organization (GSO). In addition, the number of Codex Alimentarius Commission member states increased between 2002 and 2022 from 168 to 189 (Figure 1.4). Thus, the majority of countries in 2022 have some form of limits on AFs, either by setting national standards or by proxy.

In non-tropical and developed countries where AF contamination is less prevalent, AF regulation levels are considerably more stringent compared to the regulations in tropical and developing nations, which are significantly more impacted by AF contamination (Sirma et al., 2018). For example, for the countries of the European Union, the European Commission has defined some of the strictest limits with maximum permitted levels of 2 (AFB1) and 4 µg kg^{-1} (sum AFB1, AFB2, AFG1, AFG2) and for maize and peanuts. On the other hand, the maximum levels set by the Ministry of Agriculture of the Republic of Indonesia are 15 (AFB1) and 20 µg kg^{-1} (sum AFB1, AFB2, AFG1, AFG2) for maize and peanuts, respectively. These differences have significant implications for global trade and public health risks, as outlined in the previous Chapter (Chapter 1.1.3).

Current aflatoxin limits, designed primarily for formal markets, have limited ability to protect consumers in informal settings, particularly in subsistence agriculture, which is the predominant food production system in Sub-Saharan Africa (Nji et al., 2022b). Therefore, it is important to take proactive measures to prevent aflatoxin contamination rather than relying solely on the effectiveness of limits. Such a preventive approach can protect consumers and effectively mitigate economic losses. Since AF contamination can occur at various stages of the food production chain (Chapter 1.1.2), prevention includes both pre- and post-harvest measures to ensure a comprehensive approach to controlling the risk of aflatoxin contamination.

1.1.5 Aflatoxin Prevention from Field to Fork: Integrated Approaches Along the Food Production Chain

Aflatoxin control strategies rely on the knowledge of critical factors leading to increased growth and/or AF production of mold fungi (Figure 1.2, Chapter 1.1.2). Based on sound experimental evidence, the Codex Alimentarius Commission has established several codes of practice for the prevention and mitigation of AF contamination in various crops, such as cereals (CAC, 2003), peanuts (CAC, 2004), tree nuts (CAC, 2010), figs (CAC, 2008) and spices (CAC, 2017) based on Good Agricultural Practice (FAO, 2003). These guidelines suggest management practices for all stages of the food production chain, including pre-harvest, harvesting and post-harvest stages.

In the pre-harvest stages, AF control strategies focus on improving plant health and impairing the growth and AF production of the fungi. The susceptibility of crops to fungal infection is closely related to their physiological condition, which can be influenced by various agricultural practices. The management practices recommended by the CAC to minimize AF contamination in the field can be summarized as follows: Use of certified seeds of resistant varieties that are free of toxic fungi, plowing under/ destroying/ removing plant debris that may have served or potentially serves as substrate for aflatoxigenic fungi, timely planting to avoid heat and drought stress during seed development and maturation, avoidance of plant overcrowding by maintaining optimal plant densities, crop rotation, proper plant nutrition (fertilization and liming), avoiding drought stress (irrigation), controlling fungal vectors and plant pathogens including *Asgergilli* and parasitic fungi other than *Aspergilli* (fungicides), nematodes (nematocides), mites (acaricides), insects (insecticides) and weeds (herbicides).

Recommended harvesting techniques are based on selecting harvesting conditions that reduce the risk of biological contamination. Firstly, appropriate harvest timing must be selected, which involves harvesting the crop when it has reached full maturity, ensuring acceptable moisture content, and prior to the onset of extreme weather conditions such as excessive heat, rainfall, or drought. In addition, functional and clean harvesting equipment should be used to allow timely harvesting, minimize physical damage to the harvested crop, and prevent the carryover of soil, dirt, dust, or contaminated plant material that could potentially serve as an inoculum for aflatoxigenic fungi. Any contact between the harvested crop and materials that may contain viable fungal structures should be avoided. Special

attention should be paid to individual plants that have been damaged by pests or plant fractions with visible fungal contamination. These plants should be harvested separately to prevent rapid colonization by aflatoxigenic fungi during subsequent steps such as storage, transport and processing.

Post-harvest measures focus on preventing fungal invasion and/or creating conditions unsuitable for fungal growth and toxin formation. This includes avoiding piling, heaping, and storage of freshly harvested commodities with high moisture for extended periods of time. The crop should be dried as soon as possible after harvest in a manner that minimizes damage to the grain and maintains moisture levels lower than necessary for fungal growth during storage. In cases where immediate drying is not feasible, adequate aeration should be implemented. The drying, storage, and transport processes should take place in a clean, intact, protected, dry, and well-ventilated environment to protect the commodity against rain, dew, soil, pests, bird droppings, and other potential sources of contamination. Proper cleaning of the harvest is essential to remove damaged and immature plant material, as well as other foreign matter that may pose a risk of fungal infection. Continuous monitoring of the condition of stored and transported material is necessary to maintain acceptable temperature and moisture levels and minimize the presence of rodents and stored product pests, as these conditions can create favorable conditions for mold growth and AF production. The use of approved fumigants or insecticides may be appropriate for extended periods of transportation or storage.

Although soil is the natural habitat of AF producing fungi (Horn, 2003; Elmholt, 2008), none of the proposed preventive measures specifically address soil as a conservation target. In fact, the impact of preventive measures on soil AF concentrations remains unknown. Certain actions, such as the incorporation of plant residues by plowing, may potentially contribute to elevated AF input levels to soil (Fouché et al., 2020). Further, certain recommended management practices including fertilization, liming, irrigation and pesticide use can have negative effects on the integrity and functionality of the soil microbial communities (Tilman et al., 2002; Sanaullah et al., 2020), potentially leading to changes in processes involved in the formation and dissipation of AFs. This, in turn, could affect the persistence of AFs in the soil and lead to ecological imbalances that potentially pose a threat to soil health. Therefore, soil has been largely neglected despite its central function in the context of AF prevention.

1.2 Aflatoxins in the Soil Environment

1.2.1 Entry Pathways for Aflatoxins into the Soil

Natural entry pathways include *in situ* aflatoxin production in soil and plant debris, rainfall-induced washoff from fungal-infested plant material, and moldy seeds and leaves shed by plants (Figure 1.5, Elmholt, 2008; Juraschek et al., 2022; Fouché et al., 2020). Anthropogenic activities such as livestock farming (animal excretion and manure application), planting with poor seed quality (moldy seeds) and incorporation of contaminated material (crop residues, moldy silage, waste kernels) can result in inputs of aflatoxigenic fungi and AFs into the soil (Figure 1.5).

The low water solubility of -3.5 to -0.4 $\log_{10}$ mol L^{-1} and $\log K_{OW}$ value of -0.7 to 1.8 suggest that rainfall-induced washoff is a negligible pathway for AFs to enter the soil (Table 1.1). However, it should be noted that contaminated plant material may break off and fall to the ground due to the mechanical forces induced by rain, humans or animals.

Accinelli et al. (2008) found that in soil, AFs are synthesized in in the range of 10^2 (cobs

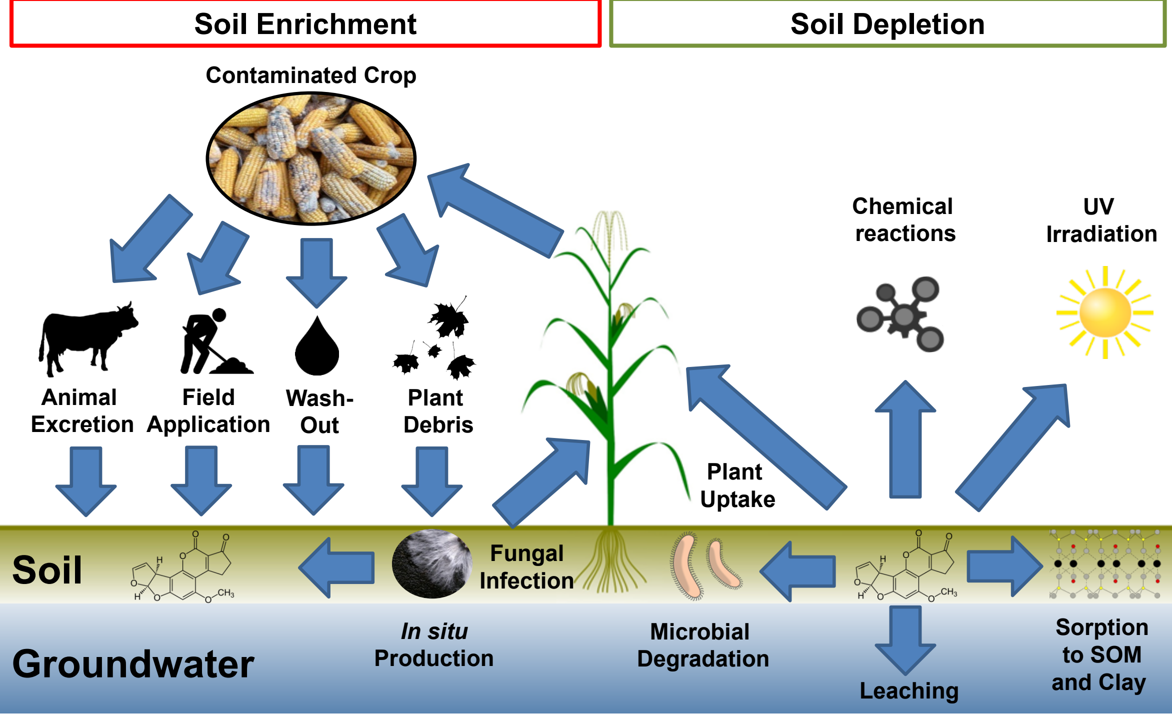

Figure 1.5: Aflatoxigenic fungi and aflatoxins in the soil environment: Possible entry pathways and degradation, sorption and transportation processes. Processes leading to soil enrichment with AFs are shown on the left side and processes leading to soil depletion are shown on the right side.

containing grain), 10^0 (leaves, stalks and cobs without grain) and 10^{-1} µg kg^{-1} (soil). These results suggest that *in situ* production in soil is rather negligible, whereas *in situ* production in plant debris (in particular nutrient rich plant material) can be a significant source of AFs in soil.

Studies from feeding trials have shown that mycotoxins from contaminated feed are transferred either as parent substance or metabolite to the urine and faeces of animals (Elmholt, 2008) and thus may enter the soil via manure. While for pig feeding trials with AFB1 contaminated feed, the excretion rates for AFB1 and AFM1 were on average 30 % (77% AFB1 + 23 % AFM1) (Thieu and Pettersson, 2009), the excretion rates were rather negligible for cows with only 1.55% (urine) and 2.79% (feces) (Allcroft et al., 1968). Thus, the excreta of certain livestock could be an important pathway for AFs to enter the soil.

Table 1.1: Physico-chemical properties and partition coefficients for the four main aflatoxins (AFB1, AFB2, AFG1, AFG2) and two main metabolites (AFB2a, AFM1). Property values represent the median of estimates from various models implemented in physical-chemical property estimation software (including OCHEM, EPISuite, ACD/Labs, and OPERA) (Tebes-Stevens et al., 2018). The individual estimates and models are given in Chapter 8.1.

Property	**Unit**	**AFB1**	**AFB2**	**AFG1**	**AFG2**	**AFB2a**	**AFM1**
Formula	-	$C_{17}H_{12}O_6$	$C_{17}H_{14}O_6$	$C_{17}H_{12}O_7$	$C_{17}H_{14}O_7$	$C_{17}H_{14}O_7$	$C_{17}H_{12}O_7$
M	g mol^{-1}	312.27	314.29	328.28	330.29	330.29	328.28
M_{mi}	g mol^{-1}	312.06	314.08	328.06	330.07	330.07	328.06
HBA	-	6	6	7	7	7	7
HBD	-	0	0	0	0	1	1
T_b	°C	474	472	511	510	509	502
T_m	°C	207	230	230	217	217	214
$log(P_v)$	mmHg	-8.9	-9.8	-10.2	-9.9	-8.5	-11.8
$Log(c_{max,w})$	mol L^{-1}	-3.1	-2.9	-3.5	-2.8	-2.6	-2.5
$Log(K_{OW})$	-	1.2	1.4	1.8	0.7	-0.4	-0.2
$Log(K_H)$	atm m^3 mol^{-1}	-9.1	-12.9	-12.3	-13.5	-17.2	-16.8
$Log(K_{OC})$	L/kg	1.9	1.9	1.8	1.8	1.2	1.3

Formula = Empiric formula; M = Molar mass; M_{mi} = Monoisotopic mass; HBA = Hydrogen Bond Acceptor Count; HBD = Hydrogen Bond Donor Count; T_b = Boiling point; T_m = Melting point; $Log(P_v)$ = Vapor pressure, logarithmic scale; $Log(c_{max,w})$ = Water solubility, logarithmic scale; $Log(K_{OA})$ = Octanol-Air-partitioning coefficient, logarithmic scale; $Log(K_{OW})$ = Octanol-Water-partitioning coefficient, logarithmic scale; $Log(K_H)$ = Henry coefficent, logarithmic scale; $Log(K_{OC})$ = Soil absorption coefficient, logarithmic scale.

1.2.2 Environmental Fate of Aflatoxins in Soil Systems

In soil, AFs may undergo various transformation, translocation and sorption processes (Figure 1.5) that depend strongly on the physicochemical properties of the soil and the functionality of the soil microbiome. However, experimental studies on the environmental fate of AFs in soil are extremely scarce and mostly originate from laboratory experiments

(Elmholt, 2008). Despite a lack of experimental studies, predictions regarding certain soil processes can be made based on the physicochemical properties (Table 1.1).

Sorption and Transport

The high boiling point of 472 - 511 °C, low vapor pressure of -11.8 to -8.5 $\log_{10}$ mmHg and Henry partition coefficient of -17.2 to -9.1 $\log_{10}$ atm m^3 mol^{-1} suggests that AFs exist solely in the particulate phase and dissipation by volatilization is very unlikely (Table 1.1).

Aflatoxins strongly sorb onto soil organic matter such as humic acids (Van Rensburg et al., 2006) with $\log K_{OC}$ values ranging from 2.80 to 3.46 (experimentally derived, Schenzel et al. (2012)) and from 1.2 to 1.8 L kg^{-1} (estimated, Table 1.1). Goldberg and Angle (1985) found that the sorption affinity of AFB1 to clay minerals was strongly dependent on clay mineral content and higher than that to soil organic matter. Moreover, the adsorption coefficient of AFB1 was about five times higher in a less humic silty clay loam (0.6% organic carbon, 37.8% clay) than in a much more humic silt loam (2.9% organic carbon, 33.6% clay).

Due to their strong interaction with clay minerals and organic carbon combined along with their low water solubility (-3.5 to -0.4 $\log_{10}$ mol L^{-1}), their mobility in water is restricted. Goldberg and Angle (1985) demonstrated a low leaching risk for various soils and found that AFB1 or its derivatives, AFB2 and AFG2, were retained in the top 20 cm of all soil types. The major part (80 to 92%) of the AFB1 applied was retained within the top 2.5 cm of soil, and no AFs were detected in the leachate.

Since AFs are intermediate-polar substances ($\log K_{OW}$ value of -0.7 to 1.8) with a low molecular weight of 312.27 to 330.29 g mol^{-1}, AFs have the potential to be taken up by plant roots and transported to aboveground plant parts. Plant uptake of AFs was documented for certain crops including lettuce (Mertz et al., 1981), maize (Mertz et al., 1980), peanut (Snigdha et al., 2013; Snigdha et al., 2015), sugarcane (Hariprasad et al., 2014), soybean (Jones et al., 1980) and several green leafy vegetables (Hariprasad et al., 2013). While significant amounts of AFs in the range between 10^0 and 10^1 µg kg^{-1} were accumulated during plant cultivation without soil, e.g. in coconut and hydroponic systems (Snigdha et al., 2013; Snigdha et al., 2015; Hariprasad et al., 2013), only small amounts of AFs of less than 1% were taken up by plants grown in soil contaminated with AFs (Mertz et al., 1980).

Biological Degradation

Microbial degradation is considered a major important removal process for aflatoxins in soil (Fouché et al., 2020). The degradation of AFs through microbial and enzymatic processes have been reported with half-lifes of a few hours to days (Wu et al., 2009; Verheecke et al., 2016). Studies were conducted in controlled environments such as bioreactors, liquid and agar cultures and media specific to the food matrix, thereby utilizing fungi from decaying wood (Alberts et al., 2009; Motomura et al., 2003), microorganisms from soil contaminated with persistent pollutants (Teniola et al., 2005; Alberts et al., 2006), microorganisms used in food processing (Megalla and Hafez, 1982), and microorganisms from the digestive tract (Kiessling et al., 1984; Jones et al., 1996). These studies reported fast AFB1 dissipation rates, occurring within a few hours to days *in vitro*. In soil, AFB1 was observed to dissipate at concentrations in the range between 10^0 and 10^4 µg kg^{-1} within a week (Accinelli et al., 2008; Angle and Wagner, 1980; Angle, 1986). So far, only mineralization has been quantitatively investigated in the context of AFB1 dissipation. Angle (1986) showed that mineralization is responsible for only a small fraction of the total dissipation of AFB1, with a 1.4 - 14% mineralization occurring over a 112-day period. However, understanding of other processes that contribute to the dissipation of AFs in soil, such as volatilization, formation of bound residues, or their incorporation into microbial biomass, and how these processes are affected by soil properties is still limited.

Abiotic Degradation

Numerous physical and chemical processes are known to detoxify AFs in food matrices, such as treatment with UV light, organic acids, ammonia, sulfites, hydroxides and peroxides, among others (Pankaj et al., 2018; Piva et al., 1995; Diao et al., 2015; Peng et al., 2018). Aflatoxins released into the soil environment may also be exposed to these conditions, although empirical evidence for such abiotic degradation processes is currently lacking. However, agricultural practices suggested by the CAC (Chapter 1.1.5) e.g. fertilization, liming, tillage and biochemical transformation reactions can introduce or form reactive substances and create conditions in the soil that may initiate abiotic degradation processes of AFs, similar to those observed in food matrices. Another aspect to be considered in the degradation process in the soil is the texture and composition, as soil components such as

clay minerals and humic substances may catalyze physicochemical degradation processes (Starr et al., 2017; Fripiat and Cruz-Cumplido, 1974; Birkel et al., 2002; Garrido-Ramírez et al., 2010; Wang et al., 2019).

Finally, it should be considered that exposure to sunlight may occur when contaminated materials are on the soil surface or in plant debris. Aflatoxins absorb light in the UV range, and treatment of contaminated foods and feeds with UV light has been reported as an efficient treatment strategy to reduce AFs levels within a very short time i.e. with half-lives of only a few hours (Diao et al., 2015). However, the abiotic degradation of aflatoxins in soil exposed to UV light has not been studied so far, though photodegradation on the soil surface may be a significant process for AF degradation in the soil environment.

1.2.3 Aflatoxins and their Potential Impact on Soil Health

Soil Health and Importance of the Soil Microbiome

Healthy soils perform crucial ecosystem functions, including water regulation, nutrient cycling, habitat provisioning, functioning as an environmental buffer or filter medium, carbon sequestration, supporting plant growth, providing physical stability and support, as well as contributing to resistance and resilience of terrestrial ecosystems (Maikhuri and Rao, 2012; Lehmann et al., 2020; Doran and Zeiss, 2000). Understanding the key characteristics of soil health is essential for managing soils in a way that supports healthy plant growth and a diverse array of soil organisms (Lehmann et al., 2020). Soil health indicators are classified as physical, chemical or biological. However, the boundaries between these categories are often blurred because many properties are due to multiple processes. For example, plant available phosphate is considered a chemical indicator, but it mainly results from microbial mineralization and plant uptake, which are biological processes (Lehmann et al., 2020). The multifunctionality and diversity of soil require multiple indicators to be quantified and integrated into an index. Soil microorganisms deserve special attention in this context. Soil microbial communities are involved in the provision of resources such as food, water, fiber, fuel, genetic resources, chemicals, medicines, and pharmaceuticals. By producing enzymes, soil microorganisms are the main drivers of the decomposition of organic matter (Hättenschwiler et al., 2005; Prasad et al., 2020), as well as secondary metabolites such as mycotoxins (Juraschek et al., 2022), xenobiotic substances, such as pesticides (Satish et al.,

2017). They are present in vast quantities, have high cumulative weight and activity, and meet most requirements to serve as valuable indicators of soil health (Saccá et al., 2017): They are sensitive to land management practices and environmental changes ("sensitive" criteria), exhibit a strong relationship with soil functions ("relevant and conceptual" criteria), and effectively illustrate the direct cause-and-effect relationship between land management decisions and plant health and productivity ("informative and interpretational" criteria) and are easily understood by land managers and are simple and inexpensive to measure ("effective and practical" criteria) (Saccá et al., 2017; Doran and Zeiss, 2000).

The most extensively studied microbial parameters for soil health assessment to date primarily include microbial biomass, respiration rates, and growth characteristics observed on agar media. However, assessment of microbial biomass and activity may not be sufficient to evaluate environmental change, as significant shifts in microbial community structure have been observed without concomitant changes in microbial biomass and activity (Joergensen and Emmerling, 2006; Fließbach and Mäder, 2004). In order to fully understand the potential impact of land management practices and environmental changes on soil health, it is important to investigate other microbial endpoints that are capable of reflecting more complex processes and functions in soil (Joergensen and Emmerling, 2006). One approach for evaluating the physiological and/or taxonomical structure of the microbial community is through the use of biomarkers such as the Phospholipid-Fatty-Acid-Analysis (PLFA) or molecular genetic measures such as amplicon sequencing and quantitative PCR with primers specific to certain taxa or functional groups. Changes in the physiological and functional state of the microbiome can be evaluated via enzymatic or respiration induced by readily available substrates such as glucose. By extending substrate-induced respiration to multiple, structurally diverse substrates, utilization patterns of carbon sources can be determined, providing valuable insights into the ability of the soil microbiome to metabolize carbon sources of varying origins and structural complexity (Campbell et al., 2003; Chapman et al., 2007). Furthermore, microbial and ecophysiological ratios can be calculated from biomass, activity and nutrient properties to detect microbial stress and changes in the composition or physiological state of the microbial community (Joergensen and Emmerling, 2006; Blagodatskaya and Kuzyakov, 2013).

Ecological Function of Aflatoxins in the Soil and Implications for the Soil Microbiome

Aflatoxigenic fungi do naturally occur in soil and plant residues as their natural habitat (Horn, 2003; Jaime-Garcia and Cotty, 2004; Orum et al., 1997; Accinelli et al., 2008). The major part of the life cycle of these fungi takes place in the soil as they not only colonize living plant tissue but also grow saprophytically on organic residues in the soil (Abbas et al., 2009). These residues serve as a reservoir for the fungus, allowing it to overwinter, and under favorable conditions resume growth with the potential to infest plants and crops (Horn, 2003; Abbas et al., 2009). In subtropical regions, aflatoxin-producing fungi and AF are natural components of soil ecosystems. To maintain a stable equilibrium in this ecosystem over the long term, there must be a balance between the natural production of AFs and their depletion. However, anthropogenic activities such as the disposal of contaminated crop residues in the field or livestock rearing can lead to additional inputs of aflatoxin-producing fungi and aflatoxins that far exceed natural levels (Chapter 1.2.1). This form of anthropogenic input could disturb the natural balance of inputs and outputs, altering both the concentration and duration of exposure of the soil microbiome to aflatoxins (Fouché et al., 2020). However, to determine if there is a potential threat to soil health, it is necessary to understand the ecological role of aflatoxins for the producing fungus and the potential impact on other microorganisms.

Aflatoxins are secondary metabolites and their production is therefore not constitutive, but depends on environmental conditions (Carter et al., 2002). During establishment and growth, aflatoxigenic fungi compete for resources with other living organisms in the soil. Therefore, aflatoxin production may be a protective response to microbial competition or predation, though empirical evidence for this function in the soil environment is limited. The presence of high AF concentrations in fungal structures that enter the soil, i.e., sclerotia, conidia spores and hyphae in infected plant material (Wicklow and Shotwell, 1983), however, suggests a protective measure by the fungus against predators and competitors during soil invasion. Furthermore, the presence of soil microbes, such as Gram-positive and Gram-negative bacteria, yeasts, and filamentous fungi, was found to enhance the *in vitro* production of AFs (Weckbach and Marth, 1977; Cuero et al., 1987; Wicklow et al., 1980). However, other studies have reported different results, with the presence of filamentous fungi and Gram-positive bacteria not affecting, reducing, or completely inhibiting AF production (Weckbach and Marth, 1977; Wicklow et al., 1980). Single species toxicity tests performed *in*

vitro on agar plates supplemented with AFB1 (30 and 100 mg L^{-1}) showed growth inhibition for some Gram-positive bacteria, including *Bacillus*, *Nocardia*, *Clostridium*, and *Streptomyces* (Burmeister and Hesseltine, 1966; Arai et al., 1967). No effects on growth were observed for other common Gram-positive and Gram-negative bacteria, fungi, algae, and protozoa. Such selective growth inhibition effects could potentially reduce the competitiveness of the affected microbes within a soil microbial community, alter the community structure of the soil microbiome, and compromise the soil functions provided by these microorganisms. In a study by Angle and Wagner (1981), the whole soil microbiome of a silt loam soil was isolated and then cultivated on AFB1 supplemented agar media (1-10,000 µg L^{-1}). At a concentration of 10,000 µg L^{-1}, AFB1 caused a 38% reduction in the number of fungi and a 34% reduction in the number of bacteria and actinomycetes, compared to the control.

However, all these studies were performed under optimized *in vitro* conditions without considering soil as an environmental matrix. Although *in vitro* studies provide key evidence on specific responses, they may not be representative of complex environmental systems since other influencing external factors are excluded (Drott et al., 2019). In addition, less than 1% of the total microbiome can be cultured on agar media (Pham and Kim, 2012). Furthermore, relatively high concentrations of aflatoxins are often used in *in vitro* bioassay studies. The environmental media contaminated with AFs are likely usually much lower. For example, AF concentrations ranging from 10^{-2} and 10^{2} µg kg^{-1} have been reported for agricultural soils and crop residues (Accinelli et al., 2008). In this regard, Drott et al. (2019) investigated the fitness of aflatoxigenic and nonaflatoxigenic isolates of *Aspergillus flavus* via quantitative PCR in soil microcosms. They found that aflatoxigenic isolates had lower fitness in natural soils across different temperature regimes (25, 37, 42 °C) and the addition of aflatoxin (500 µg kg^{-1}) to the soils did neither affect *A. flavus* growth nor the species richness of the fungal and bacterial communities (assessed via amplicon sequencing). However, it should be noted that although no lethal effects occurred, the physiology of the microbiome may have been impaired, e.g. in enzymatic or respiratory activity. In this context, Angle and Wagner (1981) artificially contaminated soil with AFB1 in a range of 1-10,000 µg kg^{-1} and determined soil respiration rates and numbers of viable fungi, bacteria, and actinomycetes of the isolated soil microbiome. Dose-dependent negative effects on the number of viable microorganisms were observed two weeks after AFB1 treatment, which persisted for almost six weeks. In addition, the authors observed a significant reduction in the respiration rate of the entire soil

microbiome at the highest AFB1 enrichment level of 10,000 µg kg^{-1} compared to the control, while respiration at lower levels was not significantly different from the control.

The influence of soil composition on the toxicity and bioavailability of AFs to the soil microbiome has not yet been systematically investigated. In soil, there are structures such as humus and clay minerals that could bind aflatoxins (Chapter 1.2.2) and thus reduce their bioavailability to microbes. Furthermore, other measures for soil health assessment such as carbon source utilization patterns and PLFA have not been widely applied in aflatoxin exposure studies. This may be due in part to the fact that previous studies on the effects of AFs on the soil microbiome were conducted prior to the availability of these methods. As a result, there is a gap in the knowledge regarding the microbial responses to AF exposure using endpoints with higher complexity and resolution, which would enable a more comprehensive assessment of the ecological function of aflatoxins for the producing fungi and their implications for the soil microbiome and associated functions.

1.2.4 Status and Challenges in the Analysis of Aflatoxins in Soils

Understanding the occurrence and fate of AFs in the environment requires the use of appropriate and reliable analytical techniques. These techniques should be applicable not only at the level of agricultural products but also in relation to the preceding steps in the production of raw materials, especially the interactions between the plant and soil ecosystems. Therefore, reliable analytical tools for the detection and quantification of AF in soils are essential for a better understanding of the environmental fate and ecological relevance of AFs in the soil environment. While a large number of methods exist for the extraction and determination of AFs in food and plant matrices, there is a lack of methods for soils.

Although previous studies reported contamination of soil with AFs ranging from 10^{-2} to 10^{1} µg kg^{-1} (Accinelli et al., 2008), it is important to note that these values may not accurately represent real environmental concentrations due to the lack of systematic validation for the specific soils analyzed using the presented analytical method. So far, reported recoveries were generally very low or methods were not systematically validated (Table 1.2). Furthermore, exorbitantly high AF concentrations of 10^{3} and 10^{5} µg kg^{-1} were applied to soil for spike-recovery experiments, which may be far above naturally occurring levels (Chapter 1.2.1).

Table 1.2: Previously described analytical procedures for the extraction of AFs from soil matrices.

Extraction technique	Extraction solvents	Extraction procedure	Soil type	AFs range (µg kg-1)	Recovery (%)	Reference
SLE	H_2O:EtOAc (1:3)	overnight shaking	silt loam	10^{1}	NA	Accinelli et al. (2008)
SLE	ACE	30 min shaking	silt loam	10^{4}	18	Angle and Wagner (1980)
SLE	$CHCl_3$, MeOH, $CHCl_3$:MeOH (80:20)	NA	loam soil	10^{4} - 10^{5}	<1	Mertz et al. (1981)
SLE	ACE	saturation with H_2O, 5 min blending	silt loam, sandy loam, clay loam, silty clay loam	10^{3}	70	Goldberg and Angle (1985)
SFE	MeCN + 2% AcOH	15 min static time	silt loam	10^{3}	72	Starr and Selim (2008)

SLE = Solid-liquid-extraction; SFE = Supercritical-fluid-extraction; EtOAc = Ethyl acetate; ACE = Acetone; MeOH = Methanol; AcOH = Acetic acid.

The challenges associated with the extraction of AFs from soil may be attributed to the complex and heterogeneous nature of soil as an environmental matrix, the way AFs interact with soil fractions and the need for detection of trace amounts, making it difficult to apply general methods (Fouché et al., 2020). In this context, soil organic carbon content and texture play a crucial role (Chapter 1.2.2), as evidenced by previous studies indicating medium-strong interactions with soil organic matter (Schenzel et al., 2012; Van Rensburg et al., 2006) and very strong interactions with clay minerals (Kang et al., 2016; Goldberg and Angle, 1985). AFs exhibit strong H-bond acceptor properties with H-bond acceptor counts of 6 - 7 and weak H-bond donor properties with H-bond donor counts of 0 - 1 (Table 1.1). Due to these strong H-bond acceptor properties, AFs strongly sorb to clay minerals via electron-donor–acceptor interactions between the two electron-rich carbonyl groups in the coumarin structure of the AFs and electron-deficient or positively charged species located at the negatively charged surface of clay minerals (Kang et al., 2016). Most solvents tested to date have been monopolar solvents that have weak H-bond acceptor properties (e.g., chloroform) or bipolar solvents (e.g., methanol) and thus may not have been able to effectively displace AFs from cation H-bond sites. Thus, it remains to be determined whether the use of solvents that have similar polarity and H-bond acceptor properties to aflatoxins can effectively overcome the strong interactions between aflatoxins and soil.

1.3 Open Questions and Rationale of the PhD Project

Currently, the study of aflatoxins primarily revolves around their potential to contaminate food and feed, rendering them unfit for human consumption and trade. This emphasis is evident in the extensive research conducted on aflatoxins and aflatoxigenic fungi, exploring their chemistry, human toxicity, and the factors contributing to their presence in food and feed, primarily during post-harvest stages (Fouché et al., 2020). Meanwhile, their relevance at pre-harvest stages as environmental micropollutants in their natural habitat, namely soil, remains largely unexplored. The environmental fate of aflatoxins in soil and the consequences of aflatoxin contamination for soil microorganisms that perform essential ecological functions remain unclear. The current limited knowledge of the occurrence, fate, and ecological consequences of aflatoxins in soil hampers the understanding of their environmental relevance and may impede the development of effective pre-harvest strategies to control aflatoxin contamination.

These knowledge gaps regarding aflatoxins in the soil are probably attributed to methodological challenges encountered in successfully extracting aflatoxins from soils. Although sorption to certain soil compartments, particularly organic matter (Schenzel et al., 2012) and clay minerals (Kang et al., 2016), is known to contribute significantly to the particularly strong interactions of AF with soil, it is not yet known how these interactions could be overcome to allow effective extraction of aflatoxins from soils. In this regard, it remains uncertain whether the particular strong electron-donor-acceptor interactions between aflatoxins and the positively charged layers of the clay minerals can be effectively counteracted by utilizing solvents with similar polarity and H-bond acceptor properties to the aflatoxins, such as acetonitrile. Overcoming these interactions in developing a suitable analytical method for the extraction of aflatoxins is an essential prerequisite for studying the occurrence and fate of AFs in the environment.

Despite a basic understanding of the pathways by which aflatoxins could possibly enter the soil (Chapter 1.2.1), there is a notable lack of experimental evidence on their general occurrence and distribution in the field, as well as on their dependence on site conditions, soil properties, and agricultural practices. Although preventive measures to successfully control aflatoxin contamination of field crops prior to harvest are well documented, their effect on aflatoxin occurrence in the soil remains uncertain. This is relevant because if such

pre-harvest measures have an impact on soil contamination, this could also have an impact on the integrity and functionality of the soil microbiome and thus soil health.

Although laboratory experiments conducted in the absence of soil have led to a general understanding of the mechanisms of aflatoxin degradation (Chapter 1.2.2), the understanding of the fate of aflatoxins in their complex natural environment "soil", is still largely incomplete. In the limited number of studies conducted in soil, rapid dissipation has been observed (Accinelli et al., 2008; Angle and Wagner, 1980; Angle, 1986), but the processes responsible for this dissipation in interaction with soil physicochemical properties and aflatoxin concentrations, remain poorly understood. Furthermore, although aflatoxins can be exposed to UV light via sunlight at pre-harvest stages, the extent to which photolytic degradation occurs in a soil matrix has not yet been tested. This is relevant as this degradation process has the potential to be a key driver in the reduction of aflatoxins in soil, consequently influencing the overall equilibrium within the soil.

In vitro studies conducted without soil have shown the toxicity of aflatoxins to specific isolated soil microbes (Chapter 1.1.3), but the potential implications for the soil microbiome, influenced by the soil matrix and available concentration, are not well understood. In addition, previous research primarily concentrated on general microbial endpoints, such as the effects of AFs on specific organism groups and their biomass and activity. However, these general biomarkers may not be sufficient to detect environmental changes, as significant changes in microbial community structure have been observed without concomitant changes in microbial biomass and activity. Such a microbial change at the community and physiology level has the potential to impair functions provided by the soil microbiome with unknown consequences for soil health and agricultural productivity.

Summarizing, in this thesis, it is attempted to address the following open research questions:

(1) How can the interactions between aflatoxins and soil sorption sites, namely clay minerals and organic carbon, be effectively overcome to enable the extraction of aflatoxins from soils?

(2) What is the occurrence and distribution of aflatoxins in contaminated soils, and to what extent does this depend on soil properties and agricultural practices intended to control fungal infestation and mycotoxin contamination of crops at the preharvest stage?

(3) Which processes contribute to the dissipation of aflatoxins in soil, and how are they modulated by soil properties, especially clay content, and aflatoxin concentration?

(4) How does aflatoxin contamination affect the soil microbiome and its associated functions, and to what extent are these effects modulated by soil properties?

1.4 Objectives, Hypotheses and Structure of the Thesis

The main objective of this PhD project was to scrutinize the mechanisms and extent of the occurrence of aflatoxins in soil, the processes of their dissipation and their impact on the soil microbiome and associated soil functions, and how these relate to soil properties. There is evidence that aflatoxins strongly interact with certain soil structures, in particular with clay minerals, affecting their mobility and availability. Therefore, I hypothesize that the clay content affects the processes of aflatoxin dissipation and the impact of aflatoxins on the soil microbiome and associated functions. Various effective pre-harvest prevention measures have been reported to control aflatoxin contamination in crops, involving inhibiting aflatoxin-producing fungi growth and production, controlling fungal vectors like insects, and enhancing plant health to reduce susceptibility to fungal infection. As a result, I hypothesize reduced aflatoxin soil levels in fields where these measures are implemented. To address these hypotheses, the structure of the PhD thesis encompasses four parts, comprising laboratory and field experiments (Figure 1.6).

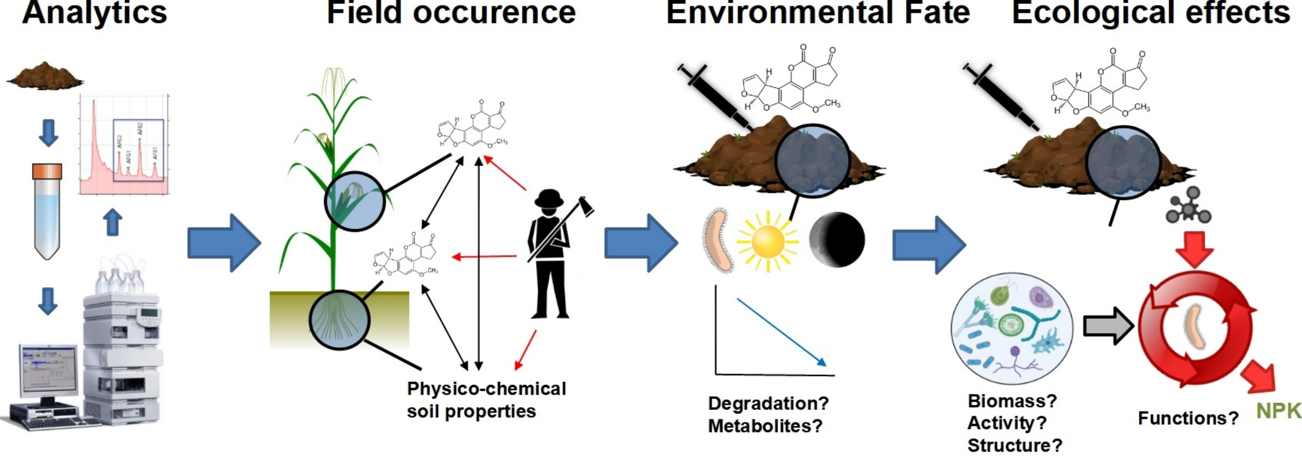

Figure 1.6: Graphical representation of the structure of the thesis.

The first part (Chapter 2) describes the development and validation of an analytical methodology capable of reliably determining AFs in the soil environment. At the start of the PhD project, the lack of appropriate analytical methods to accurately quantify aflatoxins in soil posed a significant obstacle to adequately address the research questions and hypotheses. Therefore, I first started with the development and validation of an analytical method aimed to be fast, simple, sensitive, and selective to allow for an effective analysis of the major relevant aflatoxins AFB1, AFB2, AFG1, and AFG2 in agricultural soil. The analytical method being developed for this PhD project emphasizes capacity building by avoiding the need for costly purification techniques like IAC cleanup and advanced instrumentation such as LC-MS and instead utilizing HPLC-FLD for analysis (Chapter 1.2.4). My hypothesis is that by utilizing solvents with similar characteristics to aflatoxins, namely intermediate polarity and H-bond acceptor properties (Chapter 1.2.4), and incorporating an ultrasonication treatment during solvent extraction (USE) to reduce the size of soil agglomerates and clay minerals, the strong interactions between soil sorption sites (i.e. clay minerals and soil organic carbon) and AFs can be effectively overcome.

In the second part (Chapter 3), the previously validated method was then used to analyze real soil samples to identify conditions and agricultural practices leading to elevated AF concentrations in soil. As part of the interdisciplinary and international project "AflaZ", a comprehensive field study was carried out during harvest season in a high-risk model region for Sub-Saharan Africa, namely Kenyan maize fields in the Makueni region. The investigation focused on whether various agricultural practices can effectively lower the concentration of AFs in fields. The implementation of innovative farming practices aimed at reducing the overall occurrence of aflatoxigenic fungi and decreasing the susceptibility of crop plants to fungal infestation. These practices included: (1) push-pull farming, which involves planting repellent and pest-insect attracting plants to reduce overall insect damage to crops; (2) conservation tillage, which promotes beneficial soil organisms, improves plant health, and suppresses aflatoxigenic fungi; (3) the use of non-toxigenic and mycophagic *Trichoderma* fungi, which preys on toxigenic *Aspergillus* fungi; and (4) a control group that followed conventional farming practices, as typically practiced by the local population.

In the third part (Chapter 4), a laboratory incubation experiment was carried out to investigate the underlying degradation processes and their relationship with the physicochemical properties of the soil and available concentrations of AFs. In this regard, I aimed to simulate

conditions, aflatoxins may be subjected to in fields. For this purpose, I designed a laboratory degradation experiment with two reference soils (clay and sandy loam) covering a range of soil properties, that are likely affecting the availability of AFs for the degradation processes, namely soil organic carbon and clay content. Non-sterile soils were incubated in the dark to assess the microbial degradation, while sterile soils functioned as a sterile control. Sterile soils were irradiated with UV light to simulate sunlight-induced photodegradation. Aflatoxin B1 was used as a model compound since it is the most frequently detected AF in plant-based foods and feeds and due to its toxicological relevance. The samples were further analyzed for the formation of the previously described metabolites in soil matrices, i.e. AFB2, AFB2a, AFG1 and AFG2, thereby evaluating the transformation processes of AFB1.

In the fourth part (Chapter 5), I conducted a laboratory incubation experiment to evaluate the consequences of aflatoxin exposure to the soil microbiome and associated soil functions. To comprehensively evaluate the effects at different levels, including microbial responses in terms of biomass, activity, and catabolic functions (Chapter 1.2.3), I conducted an incubation study with two reference soils that exhibited a range of physicochemical properties and were artificially contaminated with AFB1 in an environmentally relevant range. To establish links to the findings from Chapter 3, I employed the same reference soils, model compound, and concentrations.

Finally, in the concluding discussion (Chapter 6), I address the central hypothesis of this thesis, explore in detail the implications of my findings, highlight new and unanswered questions and future perspectives, and explore possible research connections.

Chapter 2

Analysis of Aflatoxins in Soil and Food Matrices

This chapter is based on: Albert, J., More, C. A., Dahlke, N. R. P., Steinmetz, Z., Schaumann, G. E., and Muñoz, K. (2021). "Validation of a Simple and Reliable Method for the Determination of Aflatoxins in Soil and Food Matrices". *ACS Omega* 6.29, pp. 18684–18693. DOI: 10.1021/acsomega.1c01451

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Validation of a Simple and Reliable Method for the Determination of Aflatoxins in Soil and Food Matrices

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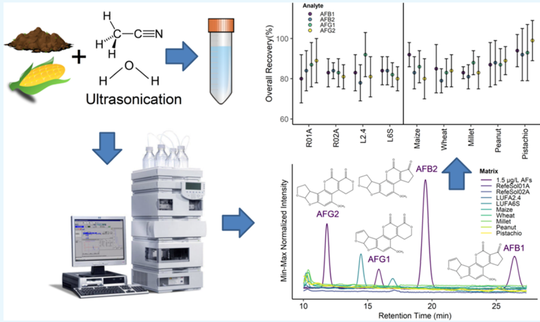

ABSTRACT: Aflatoxins (AFs) are toxic fungal secondary metabolites that are commonly detected in food commodities. Currently, there is a lack of generic methods capable of determining AFs both at postharvest stages in agricultural products and preharvest stages, namely, the agricultural soil. Here, we present a simple and reliable method for quantitative analysis of AFs in soil and food matrices at environmentally relevant concentrations for the first time, using the same extraction procedure and chromatography, either by HPLC-FLD or LC−MS. AFs were extracted from matrices by ultrasonication using an acetonitrile/water mixture (84:16, v + v) without extensive and time-consuming cleanup procedures. Food extracts were defatted with *n*-hexane. Matrix effects in terms of signal suppression/enhancement (SSE) for HPLC-FLD were within ±20% for all matrices tested. For LC−MS, the SSE values were mostly within ±20% for soil matrices but outside ±20% for all food matrices. The sensitivity of the method allowed quantitative analysis even at trace levels with quantification limits (LOQs) between 0.04 and 0.23 $\mu g\ kg^{-1}$ for HPLC-FLD and 0.06−0.23 $\mu g\ kg^{-1}$ for LC−MS. The recoveries ranged from 64 to 92, 74 to 101, and 78 to 103% for fortification levels of 0.5, 5, and 20 $\mu g\ kg^{-1}$, respectively, with repeatability values of 2−18%. The validation results are in accordance with the quality criteria and limits for mycotoxins set by the European Commission, thus confirming a satisfactory performance of the analytical method. Although reliable analysis is possible with both instruments, the HPLC-FLD method may be more suitable for routine analysis because it does not require consideration of the matrix.

1. INTRODUCTION

Aflatoxins (AFs) are secondary metabolites produced by certain molds of the genus *Aspergillus* that are widespread in crops and food commodities. AFs are toxic and carcinogenic to humans and therefore, their occurrence is associated with serious health concerns. As a consequence, maximum limits have been set in foods for the main AFs B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2) in order to protect consumers against dietary exposure. Commodities exceeding the maximum levels cannot be further commercialized, leading to substantial economic losses for agriculture and livestock farmers. At present, AFs are almost exclusively studied for their potential to contaminate food and feed, which is reflected in the overwhelming research on AFs and aflatoxigenic fungi with regard to their chemistry, and the causes of their occurrence in feed and food and to the toxic effects that they may exert on humans and animals.[1] In order to understand the environmental occurrence and fate of AFs, suitable and reliable analytical methods are required. These methods should be accessible not only at the level of the agricultural product but also considering previous steps in the production of commodities, namely, the plant−soil ecosystem. Soil is considered the natural habitat for aflatoxigenic fungi and serves as a reservoir for primary inoculum in plant infestation.[2] For this reason, the development and validation of analytical tools which investigate the potential of soil as a mycotoxin source are imperative.

The contamination levels of AFs reported in agricultural soils ranged from 10^{-2} to 10^{1} $\mu g\ kg^{-1}$.[4] These levels however may not represent environmental concentrations since the described analytical method has not been subjected to a systematic validation in terms of sensitivity, accuracy, and matrix effects. Other presented recovery rates for soil matrices were either not suitable[4,5] or the procedures were not systematically validated[3,6,7] (Table 1). In addition, AF

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Table 1. Previously Described Methods for the Extraction of AFs from Soil Samples[a]

extraction technique	solvents	extraction procedure	soil type	clay (%)	C_{org} (%)	fortification level (μg kg^{-1})	recovery (%)	references
solvent extraction	acetone	30 min shaking	silt loam	22.2	2.4	1×10^4	18	Angle & Wagner[5]
solvent extraction	chloroform, MeOH, chloroform/MeOH (80:20)	NA	loam soil	28.1	NA	$3.3–26.7 \times 10^4$	<1	Mertz et al.[4]
solvent extraction	acetone	5 min blending	silt loam	33.6	2.9	5.7×10^3	70	Goldberg & Angle[9]
			sandy loam	12.1	1.5			
			clay loam	27.5	1.8			
			silty clay loam	37.8	0.6			
supercritical fluid extraction	acetonitile +2% acetic acid	15 min static time	silt loam	58.5	1.87	1.7×10^3	72	Starr & Selim[8]
solvent extraction	water/ethyl acetate (1:3)	overnight shaking	silt loam	8.1−8.3	0.47−0.55	10	NA	Accinelli et al.[7]

[a]NA = not available, C_{org} = soil organic carbon content.

fortification levels were in the range of $10–10^5$ μg kg^{-1},[4,5,8,9] which may be far above the environmentally relevant levels (Table 1). A probable reason for the lack of validation proceeding may be attributed to the complexity of soil as the environmental matrix and to the interaction of AFs with soil fractions.[1] In this context, the soil organic matter and soil texture are of particular importance since AFs strongly sorb to soil organic carbon[10,11] and clay minerals.[4,5,12,13] This methodological challenge may be overcome by weakening the chemical interactions between the matrix and AFs via introduction of additional extraction procedures to further facilitate the transition of the analytes into the liquid phase. The introduction of an additional ultrasonication step during solvent extraction (USE) is reported to minimize solvent consumption while improving the extraction efficiency for many substances.[14] As far as we know, a USE method for the extraction of AFs from soil matrices has not yet been reported. However, due to the limited selectivity of USE, a high load of matrix components is simultaneously extracted with the analytes. Such coextracted matrix components can heavily affect the analytical performance of the detection method, which is why USE methods are often used in combination with further cleanup steps.[14] For analysis of AFs, liquid chromatography with mass spectrometry (LC−MS) and fluorescence detection (HPLC-FLD) are the methods of choice.[15,16] Both methods are however prone to interferences with coeluting matrix components, affecting both the separation step and the intensity of the detection response. In case of LC−MS, such coeluting matrix components strongly affect the ionization efficiency of the target analytes, resulting in either a loss or an increase in response. This matrix effect must be evaluated when validating a method to avoid over- or underestimation of the concentration.[17] If such matrix components also emit fluorescence at the wavelengths of the target analytes and are not sufficiently separated from the target peaks, such coeluting matrix components may cause a false-positive result. Current methods used in AF analytics to overcome matrix interactions and effects are solid-phase extraction with silica gels, imprinted polymers, or immunoaffinity columns.[18] However, these methods are associated with a comparatively high cost and workload, particularly in routine analysis of environmental samples. The current strong dependence of AF analysis on extensive and expensive sample purification techniques or on analytical tools such as LC−MS is a problem, particularly in countries affected by AF outbreaks.[16] Hence, there is a need for cost-efficient and simple alternative approaches.

In the present work, the suitability of a generic and proven solvent composition (acetonitrile/water, 84/16, v + v)[19−22] in combination with ultrasonication for the extraction of AFs from soils and plant-based foods is tested and validated according to the requirements of the Eurachem Guide[23] and European Commission (EC) Regulation no. 401/2006.[24] We aimed to prevent the coelution of interfering peaks by developing a suitable chromatographic method to enable analysis without extensive and time-consuming cleanup steps. To evaluate the effect of matrix composition, four different agricultural soils and five agricultural products were evaluated using USE, followed by LC−MS analysis. The optimized procedure was evaluated in terms of recovery, linearity, selectivity, precision, detection and quantification limits (LOD and LOQ, respectively), and matrix effects.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Reagents. Methanol (MeOH) and acetonitrile (MeCN) used for extraction, HPLC-FLD chromatography and preparation of standards were of the HPLC grade (Carl Roth, Karlsruhe, Germany). MeOH for LC−MS chromatography was of the LC−MS grade (Fisher Scientific, Schwerte, Germany). Ultrapure water (H_2O) was used throughout all work and was produced by a Milli-Q-water purification system (18.2 MΩ cm^{-1}, EASYpure II, Millipore Bedford, MA). A standard mix solution with certified concentrations of 20 μg mL^{-1} each for AFB1, AFB2, AFG1, and AFG2 dissolved in MeCN was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). From this standard solution, a working standard solution of an AF mixture containing 1000 μg L^{-1} of each AF was prepared in MeOH. This working standard was used for fortification of samples and preparation of calibration standards (solvent and matrix matched) in MeOH/H_2O (20:80, v + v). All solutions were stored at −20 °C in the dark until analysis.

2.2. Soil and Food Samples. Experiments were carried out using four soil types and five different food commodities to compensate for differences in matrices. RefeSol 01-A and RefeSol 02-A (Fraunhofer IME, Schmallenberg, Germany) and LUFA 2.4 and LUFA 6S (LUFA, Speyer, Germany) served as reference soils from organically managed arable areas. These soils were selected to cover a wide range of physicochemical properties, which are expected to have an influence on extraction efficiency (Table 2). The soil organic carbon and clay mineral contents, as reflected in soil texture (clay content),

are of particular interest as these soil fractions represent sorption sites for organic molecules and can thus impede successful extraction.[4,5,10−13] Soils were homogenized, air-dried, and 2 mm-sieved. The five selected food matrices included maize, wheat, millet, peanut, and pistachio. These foods were selected because of their relevance as commodities frequently contaminated by AFs. Matrices were obtained as powders at a local retail market except for pistachios and peanuts, which were mechanically ground to obtain a fine and homogenized powder prior to the extraction. All food samples were air-dried prior to extraction.

Table 2. Physicochemical Properties of the Tested Reference Soils[a]

soil	sand (%)	silt (%)	clay (%)	C_{org} (%)	pH	CEC (mequiv/100 g)
RefeSol 01-A	74	19.8	6.2	0.89	5.3	1.16
RefeSol 02-A	5.7	78.3	16.0	1.04	6.6	12.5
LUFA 2.4	32.1	41.6	26.3	1.78	7.4	24.2
LUFA 6S	23.8	35.3	40.9	1.99	7.2	23

[a] C_{org} = soil organic carbon content, CEC = cation exchange capacity.

2.3. Sample Fortification and Extraction. Fractions of 5 g (dry weight, dw) of air-dried samples were placed in 50 mL centrifuge tubes and fortified with AFB1, AFB2, AFG1, and AFG2. Three contamination levels (0.5, 5, and 20 μg kg^{-1}) were achieved via fortification with 100 μL of 0, 25, 250, and 1000 μg L^{-1} in methanolic solutions. Fortification levels were chosen to fit with the concentration levels set by Commission Regulation (EC) no. 401/2006[24] (i.e., ≤1 μg kg^{-1}, 1−10 μg kg^{-1}, and >10 μg kg^{-1}), which are used to evaluate the suitability of an analytical method in terms of recovery rates. The three fortification levels were compared with extraction without fortification. Fortified samples were vortexed for 10 s to obtain a homogeneous sample and left under the fume hood for 30 min to allow the solvent to evaporate. AFs were extracted from the samples using 15 mL of a MeCN/H_2O (84:16, v + v) mixture using an orbital shaker at 180 rpm for 30 min. The extraction was followed by 15 min ultrasonication followed by centrifugation at 2190*g* for 5 min. A 1 mL aliquot was transferred to centrifuge tubes and evaporated until dryness under a gently stream of nitrogen at 40 °C. The dried extracts were reconstituted with 200 μL of MeOH and vortexed for 10 s. The reconstituted samples were then conditioned with 800 μL water and vortex-mixed for 10 s. Aliquots of 400 μL of *n*-hexane was added to the extracts obtained from food matrices and vortexed for 10 s to remove the coextracted fat,[25,26] which may otherwise negatively affect the analytical performance.[27−29] The *n*-hexane layer was discarded. To remove undissolved particles, the conditioned samples were centrifuged at 13,000*g* for 1 min and the supernatant was transferred to HPLC amber glass vials. The filtered extracts were stored at −20 °C in dark until measurements.

2.4. LC−MS Analysis. LC−MS analyses were performed on an Exactive Orbitrap system (ThermoFisher Scientific Inc., Waltham, USA) operating in positive mode in the range of 200−500 *m*/*z*. The scan was performed in high-resolution mode corresponding to a value of 50,000 at *m*/*z* 200 at a scan rate of 2 Hz. The automatic gain control (AGC) target value was set to 1 × 10^6 (balanced). By foregoing exhaustive extract purification for matrix removal, an Orbitrap system allows for higher-resolution detection of *m*/*z* ratios. AFs were separated on a Hypersil GOLD C18 1.9 μm 1.0 × 100 mm column (ThermoFisher Scientific Inc., Waltham, USA) by gradient elution using MeOH (eluent A) and ultrapure water (eluent B), both conditioned with 0.1% formic acid and 4 mM ammonium formate at a flow rate of 0.2 mL min^{-1}. The program (i) began with an isocratic phase of 2 min at 10% eluent A, (ii) followed by a linear increase to 95% over 8 min, (iii) an isocratic phase of 3 min, (iv) a linear decrease to 10%, and (v) a reconditioning phase of 2 min. The injection volume was set at 10 μL for both sample extracts and calibration standards. AFs were measured in positive electrospray ionization mode with $[M + H]^+$ adducts. Target analysis was performed with ionic masses at 313.0715, 315.0860, 329.0650, and 331.0800 *m*/*z* for AFB1, AFB2, AFG1, and AFG2 respectively. Furthermore, the $[M + NH_4]^+$ adduct was continuously monitored alongside the $[M + H]^+$ adduct for confirmation purposes, with *m*/*z* of 330.0962, 332.1132, 351.0467, and 353.0631 for AFB1, AFB2, AFG1, and AFG2, respectively. A concentration-to-signal relationship for the $[M + NH_4]^+$ adduct, as well as the absence of a signal in the matrix blank was confirmed (Figure S2). The electronic setting was defined as follows: capillary voltage, 25 V; spray voltage, 4 kV; tube lens voltage, 75 V; skimmer voltage, 14 V; capillary temperature, 275 °C.

2.5. HPLC-FLD Analysis. The AF analyses were performed on an Agilent 1200 series (Agilent, Santa Clara, USA) system (G1311A Quaternary pump, G1322A degasser, G1329A autosampler) equipped with a column oven (Jetstream 2 column thermostat, KNAUER, Berlin, Germany), postcolumn UV-derivatization module (UVE, KNAUER, Berlin, Germany), and fluorescence detector (G1321A, Agilent, Santa Clara, USA). Chromatographic separation of AFs was achieved on a Zorbax Eclipse XDB-C18 reversed-phase 5 μm 4.6 × 150 mm column (CS Chromatographie-Service, Langerwehe, Germany) using an isocratic elution mode consisting of a mixture of H_2O/MeOH/MeCN (72:20:8, v + v + v) at a flow rate of 1.7 mL min^{-1}. The injection volume was set at 100 μL for both sample extracts and calibration standards. The fluorescence detector was set to an excitation wavelength of 365 nm and emission wavelengths of 455 nm for AFG1 and AFG2 and 435 nm for AFB1 and AFB2. The selection criteria for AFs in samples were the retention time and peak shape of the analytes observed in matrix-matched calibration solutions.

2.6. Quality Criteria. The method was tested in terms of the selectivity, linear working range, matrix effects, accuracy (trueness and precision), LOD, and LOQ in accordance with the EuraChem guide[23] to fulfill the requirements of Commission Regulation (EC) no. 401/2006.[24]

The selectivity was tested through (i) the analysis of nonfortified and fortified samples at four levels (no spike, 0.5, 5, and 20 μg kg^{-1}) via LC−MS and HPLC-FLD, (ii) matrix-matched calibration standards via LC−MS and HPLC-FLD, and (iii) identification of alleged AF peaks via *m*/*z*-ratios of adduct with the highest ($[M + H]^+$) intensity as the quantifier and the second highest intensity ($[M + NH_4]^+$) as the qualifier using high-resolution MS detection.

The linear working range was assessed through measurements of 10 calibration levels in the range of 0.01−50 μg L^{-1}. Nominal concentrations were plotted against the integrated area. A linear range between 0.05 and 10 μg L^{-1}, equivalent to 0.15−30 μg kg^{-1} dry solid matrix, was approximated by visual inspection of the scatter plot.[30] Linearity of the approximated

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working range was estimated by duplicate measurements of six calibration standards in the range between 0.05 and 10 μg L^{-1} by the use of residual plots (residuals vs predicted values) and calculation of the adjusted coefficient of determination (R^2_{adj}).[30] The assumption of normality was assessed via QQ-plots (standardized residuals vs theoretical quantiles).[30] The homoscedasticity criterion was checked via scale-location plots (square root of standardized residuals vs predicted values).[30] In case calibration data did not meet the assumption of homoscedasticity, the weighted least-squares (WLSs) linear regression model was applied as a simple and effective way to counteract the greater influence of higher concentrations on the regression model, improving the accuracy at the lower end of the calibration curve.[31] The optimal weighting factor w_i was chosen according to the procedure described by Almeida.[31] The following w_i were tested: $1/x^{0.5}$, $1/x$, $1/x^2$, $1/y^{0.5}$, $1/y$, and $1/y^2$, where x is the nominal concentration and y is the signal (i.e. peak area). In brief, the best weighting factor was chosen according to the percentage relative error (% RE), which compares the calculated concentrations x_{calc} with the nominal concentrations x for all tested weighted models

$$\mathrm{RE}\ (\%) = \frac{x_{calc} - x}{x} \times 100$$

The best w_i was that which presents the least RE_{sum} (%)

$$\mathrm{RE}_{sum}\ (\%) = \sum_{n=1}^{i} \sqrt{(\mathrm{RE}_i(\%)^2}$$

The magnitude of matrix effects was estimated by comparing the slopes of solvent (b_{sol}) and matrix-matched calibrations (b_{mm}) and quantitatively expressed as the signal suppression/enhancement (SSE) ratio using the following equation[32]

$$\mathrm{SSE}\ (\%) = \frac{b_{mm} - b_{sol}}{b_{sol}} \times 100$$

All AF concentrations were calculated using weighted matrix-matched calibration.

Trueness in terms of bias was calculated as relative spike recovery R (%) using data from spiking experiments with the following equation

$$R(\%) = \frac{X_{found}}{X_{fortified}} \times 100$$

where X_{found} is the concentration calculated using the weighted matrix-matched calibration curve and $X_{fortified}$ is the nominal added concentration. According to Commission Regulation (EC) no. 401/2006,[24] recovery rates for AFs should be in the range of 50−120, 70−110, and 80−110% for concentrations ≤1, 1−10, and >10 μg kg^{-1}, respectively.

Precision in terms of repeatability was estimated as the relative standard deviation (RSD_r (%)) of replicate measurements at each fortification level (n = 10)

$$\mathrm{RSD}_r\ (\%) = \frac{\mathrm{SD}_i}{\mathrm{mean}_i} \times 100$$

where SD_i is the standard deviation and mean_i is the arithmetic mean of respective recovery rates. According to Commission Regulation (EC) no. 401/2006,[24] the recommended maximum relative standard deviation under repeatable conditions $\mathrm{RSD}_{r,rec}$ can be calculated using following equation

$$\mathrm{RSD}_{r,rec}\ (\%) = 0.66 \times \mathrm{RSD}_{R,rec}\ (\%)$$

where $\mathrm{RSD}_{R,rec}$ is the recommended maximum relative standard deviation under reproducible conditions, which can be derived from the modified Horwitz equation[33] for concentration ratios <1.2 × 10^{-7} (i.e., 1 = 100 g/100 g, 0.001 = 1000 mg/kg)

$$\mathrm{RSD}_{R,rec}\ (\%) = 22\%$$

This results in $\mathrm{RSD}_{r,rec}$ of 14.52% for the concentrations studied. However, according to Commission Regulation (EC) no. 401/2006,[24] the maximum permitted relative standard deviation may be double the recommended value, that is, 29.04%.

The LOD and LOQ were estimated based on data of the recovery experiment. Samples fortified with 0.5 μg kg^{-1} AFs (N = 10) were used for determination of the LOD and LOQ. The absence of AFs was previously confirmed for the investigated soil and food samples. The LOD and LOQ were calculated using the following equations

$$\mathrm{LOD} = 3 \times \mathrm{SE}$$

$$\mathrm{LOQ} = 10 \times \mathrm{SE}$$

where SE is the sample standard error derived from replicate observations. The target quantification level is set to the maximum levels for certain contaminants in foodstuff intended for direct human consumption listed in Commission Regulation (EC) no. 1881/2006.[24] In brief, these limits are as follows: 2 (AFB1) and 4 μg kg^{-1} (sum AFB1, AFB2, AFG1, AFG2) for peanut, maize, wheat, millet; 8 (AFB1) and 10 μg kg^{-1} (sum AFB1, AFB2, AFG1, AFG2) for pistachio; 0.1 μg kg^{-1} (AFB1) for baby, infant, young children, and medical use. However, for soil matrices, no limits are defined yet by the EC.

2.7. Data Analysis. Data processing and statistical analyses were performed using R (version 4.0.3, R Core Team). The weighting factors for weighted calibration were selected based on the minimum RE_{sum} using the command "weight_select" (package "envalysis", available from https://doi.org/ft9p). Matrix effects in terms of SSE were calculated using the command "matrix_effect" (package "envalysis", available from https://doi.org/ft9p). Effects of the matrix type ("Matrix type"; factor with the two levels "food" and "soil") and instrumentation ("Method"; factor with two levels "LC−MS" and "HPLC-FLD") and their interaction on the absolute value of the matrix effect (SSE), LOD, and LOQ were tested using two-way ANOVA models. Effects of the matrix type ("Matrix type"; factor with the two levels "food" and "soil") and fortification level ("Fortification level"; factor with three levels "low", "medium" and "high") and their interaction on recovery (Recovery) and relative standard deviation (RSD_r) were tested using two-way ANOVA models. The significance of predictor variables was tested with an F-test. The effect of clay content ("clay") and soil organic carbon content (C_{org}) on recovery ("Recovery) was tested via linear mixed effect models with the command "lmer" (package "lmerTest", available from https://doi.org/dg3k). Because of the nested design, where recovery rates are obtained at different fortification levels, the variable "fortification level" (factor with the three levels "low", "medium" and "high") was included as a random effect. Kenward−Roger approximation was used for computing the degrees of freedom and t-statistics of the predictors of the mixed effect models (package "lmerTest", available from

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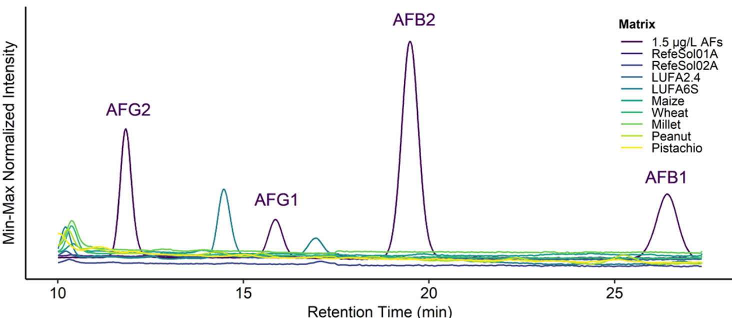

Figure 1. Extracted HPLC-FLD chromatograms obtained from injection of the solvent calibration standard at 1.5 μg L^{-1} and blanks of respective matrices (highlighted by different colors). Additional chromatograms showing sample blanks, solvent calibration standard (1.5 μg L^{-1}), matrix-matched calibration standard (1.5 μg L^{-1}), and fortified sample extracts (20 ng g^{-1}) for two food matrices (maize and wheat) and two soil matrices (RefeSol 01-A, LUFA 6S) are presented in the Supporting Information (Figure S1).

https://doi.org/dg3k).[34] Significant results ($P < 0.05$) are shown in bold. Model assumptions were verified using diagnostic plots, that is, normality of residuals was checked via QQ-plots and homoscedasticity of residuals was checked via scale-location plots (square root of standardized residuals vs predicted values).[30]

3. RESULTS AND DISCUSSION

3.1. HPLC-FLD Method Development. During development of the HPLC-FLD method, several chromatographic conditions were tested such as different combinations of H_2O/MeOH/MeCN at different column temperatures. Frequently used eluent mixtures at 55−65% H_2O and variable amounts of MeOH and MeCN[35−39] achieved baseline separation of the four investigated AFs. However, none of the tested conditions were able to baseline-separate interference peaks from the analyte peaks. To overcome the coelution problem, weaker mobile-phase compositions in terms of elution power were tested at different temperatures and flow rates. An increase in the H_2O content prolonged the run time but resulted in better separation. An increase in temperature and flow rate lowered the run time but lead to insufficient separation and a decrease in the sensitivity. Furthermore, the ratio between MeOH and MeCN considerably hampered the separation of AFG1 and AFB2. While an increase in MeCN generally resulted in faster run time, it led to poor baseline separation of the AFG1 and AFB2 peaks. Finally, a separation at 35 °C and a mobile-phase composition of 72:20:8 (v + v + v) H_2O/MeOH/MeCN at a flow rate of 1.7 mL min^{-1} proved to be a good compromise between separation performance, speed, and sensitivity. Chromatograms of all tested matrices for the optimized method are presented in Figure 1. Additional chromatograms for sample blanks, solvent, and matrix-matched calibration standard (1.5 μg L^{-1}) and fortified sample extracts (20 ng g^{-1}) for two food matrices (maize and wheat) and two soil matrices (RefeSol 01-A and LUFA 6S) are available in the Supporting Information (Figure S1).

3.2. Matrix Effects, Interferences, and Linear Working Range. Prior to the analysis of fortified samples, linear working range, matrix effects, and necessity of weighting were evaluated for all analytes in all matrices in order to determine the quantification strategy. Suitable $R_{adj}^2 \geq 0.991$ were achieved for all calibration curves in all matrices and for all analytes using the nonweighted calibration (Table S1). However, since all calibration models did not meet the assumption of homoscedasticity, WLS models were applied to improve the precision at the lower end of the calibration. Although the R_{adj}^2 values had significantly decreased by an average of −0.0072 ($p < 0.001$, paired t-test, $df = 79$, Tables S1 and S3), the application of w_i significantly reduced the RE_{sum} (%) by an average of −284% ($p < 0.001$, paired t-test, $df = 79$, Tables S1 and S3) and hence improved the precision at the lower end of the calibrations. The slope ratio of the weighted matrix matched and solvent calibration was then used to evaluate matrix effects. Matrix effects in terms of the SSE were significantly ($p < 0.001$, F-ANOVA, $df = 1$, Tables S1 and S3) lower for the HPLC-FLD (average 5 ± 4%) than for the LC−MS (average 31 ± 8%) method (Figure 2). Moreover, the interaction between instrument and matrix type was significant ($p < 0.001$, F-ANOVA, $df = 1$, Tables S1 and S3), indicating stronger matrix effects in food matrices than in soil matrices for LC−MS, while the opposite pattern was observed for HPLC-FLD (Figure 2).

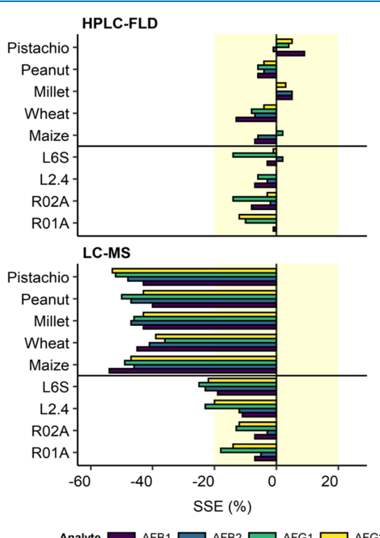

Figure 2. Matrix effects in terms of SSE for HPLC-FLD (left) and LC−MS (right). The colored band marks the threshold of ±20% to justify using solvent calibration, as opposed to the matrix-matched standard.

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SSE values ±20% are generally considered suitable values, indicating minor matrix effects and may function as a threshold to justify using solvent calibration as opposed to matrix-matched calibration as this variation would be close to repeatability values.[40,41] Overall, the matrix effects were within the ±20% range for all matrices tested via HPLC-FLD. Furthermore, matrix effects were mostly within the 20% threshold for the soil matrices tested via LC−MS. Thus, according to the suggested threshold of ±20%, it would be sufficient to use solvent calibration instead of matrix-matched calibration for concentration calculation. Since the use of a matrix-matched calibration would require an analyte-free matrix blank, the possibility of using a solvent calibration instead of a matrix-matched calibration makes the proposed method applicable to cases where no sample blank is available. All food matrices tested via LC−MS were far below the threshold of ±20%, and hence sample purification (i.e., immunoaffinity chromatography (IAC) or solid-phase extraction (SPE) procedures) or matrix-effect compensation strategies such as matrix-matched calibration and stable isotope dilution assays would be necessary.[42] In contrast, since no coeluting interferences occurred and matrix effects were almost negligible, HPLC-FLD may be more suitable for routine analyses.

3.3. Limits of Detection and Quantification. The method's LOD an LOQ ranged from 0.02 to 0.07 and 0.06 to 0.23 μg kg^{-1} for LC−MS and from 0.01 to 0.07 and 0.04 to 0.23 μg kg^{-1} for HPLC-FLD (Figure 3, Table S1). The method's LOD and LOQ were significantly ($p < 0.001$, F-ANOVA, $df = 1$, Table S1 and S3) higher for LC−MS (0.04 ± 0.01 and 0.14 ± 0.04 μg kg^{-1}) than for HPLC-FLD (0.03 ± 0.01 and 0.10 ± 0.04 μg kg^{-1}). There was no significant (p = 0.23 and 0.24, F-ANOVA, $df = 1$, Tables S1 and S3) difference between values for food and soil matrices. The significant interaction term for LOD ($p = 0.013$, F-ANOVA, $df = 1$, Tables S1 and S3) and LOQ ($p = 0.01$, F-ANOVA, $df = 1$, Tables S1 and S3) suggests higher values in food matrices than in soil matrices for the LC−MS, while an opposite pattern was observed for HPLC-FLD.

Interestingly, the method sensitivity in terms of LOD and LOQ was better for the HPLC-FLD compared to the LC−MS. This may be explained by the fact that the lower instrumental sensitivity of the HPLC-FLD (i.e., the analyte concentration-to-signal relationship) was compensated by a much higher injection volume. In LC−MS applications, smaller columns are usually used to enable separations at lower flow rates. Low flow rates are needed to ensure sufficient evaporation of the solvent after leaving the column. With HPLC, larger columns and thus higher injection volumes can be used. In addition, using the on-column focusing technique[43−45] in which the sample is prepared in a weaker solvent than the mobile phase, it was possible to greatly increase the injection volumes up to 100 μL as compared to the volumes that are usually used for such column dimensions, that is, 8−40 μL as suggested by many manufacturers. Irrespective of the instrumentation, most LOQs were around 10−300 times below the target quantitation levels based on the maximum levels for certain contaminants in foods intended for direct human consumption listed in Commission Regulation (EC) no. 1881/2006.[24] Only for foods for infants, young children, and for medical use, the LOQs for almost all matrices were above the limit value of 0.1 μg kg^{-1}. This threshold may still be achieved by concentrating the extract. However, this would significantly increase the already high

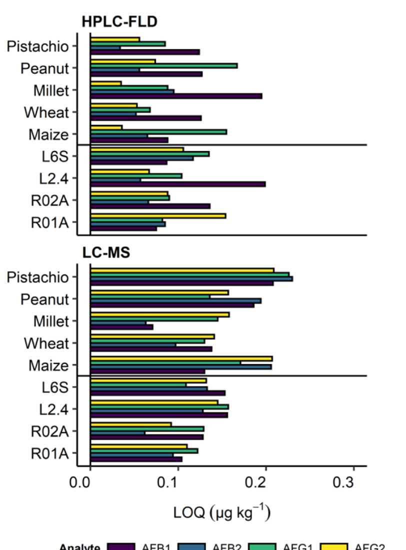

Figure 3. LOQs (method) for investigated AFs measured via LC−MS and HPLC-FLD.

matrix effects in foodstuff (LC−MS). Furthermore, it is likely that interfering peaks near the analyte peaks (HPLC-FLD) may broaden considerably due to column overloading and thus lead to an insufficient separation of analyte peaks and interfering peaks. Treatment of the extract by IAC or SPE could simultaneously concentrate the extract and purify it from matrix components so that lower LOQs could be achieved. Altogether, both methods had proven to be suitable for the monitoring of foodstuff for human consumption.

3.4. Trueness and Precision. The recovery rates ranged from 64 to 92, 74 to 101, and 78 to 103% for the fortification levels of 0.5, 5 and 20 μg kg^{-1}, respectively (Figure 4, Table S2). The recovery rates were significantly lower at the lowest fortification level ($p < 0.001$, F-ANOVA, $df = 2$, Table S2 and S3). In addition, the recovery rates were significantly (p = 0.0194, F-ANOVA, $df = 2$, Table S2 and S3) higher in food matrices than in soil matrices. Furthermore, neither clay content ($p = 0.507$, t-test, $df = 44$, Table S2 and S3) nor C_{org} ($p = 0.494$, t-test, $df = 44$, Table S2 and S3) had a significant effect on recovery rates in soil matrices. Overall, the percentage recovery rates were in accordance with the performance criteria imposed by Commission Regulation (EC) no. 401/2006.[24] Only for the clayey soil (LUFA 6S) at a fortification level of 20 μg kg^{-1}, the spike recovery of 78% is slightly lower than the proposed range of 80−110% for levels >10 μg kg^{-1}, which may not be problematic since these limits are only valid for food matrices, and so far, no limits are defined for soil matrices. However, the recovery is still fulfilling the limits for soil matrices reported in other guidelines such as the limits of 70−110% defined by the EC in the SANCO/3029/99 rev.411/07/00 guide.[46] The calculated relative standard deviations of the repeatability were in the range of 2−18% and hence below the maximum permitted relative standard deviation of 29%. Furthermore, 136 out of 144 (≙94%) matrix/fortification level/analyte combinations were below the recommended maximum relative standard deviation of 14.52%. Thereby, all exceeding values originated from the lowest fortification level of 0.5 μg kg^{-1}. In general, the RSD was

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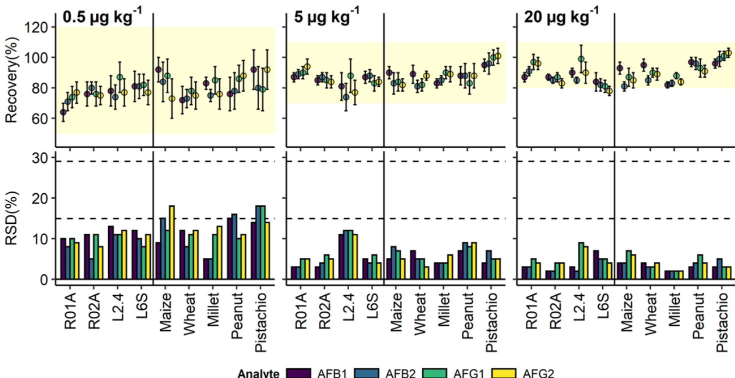

Figure 4. Trueness in terms of mean and standard deviation of spike recovery (top) and relative standard deviation of spike recovery (bottom) for the three fortification levels at 0.5 μg kg^{-1} (left), 5 μg kg^{-1} (center) and 20 μg kg^{-1} (right). Horizontal bands (top) indicate the trueness thresholds set up by the EC of 50−120% for <1 μg kg^{-1} (left), 70−110% for 1−10 μg kg^{-1} (center), and 80−110% for >10 μg kg^{-1} (right). The dashed lines are indication of the maximum recommended and maximum permitted repeatability of 14.52 and 29.04% respectively set by the EC.

significantly higher at the lowest fortification level ($p < 0.001$, F-ANOVA, df = 2, Tables S2 and S3), but no effect of matrix type ($p = 0.254$, F-ANOVA, df = 1, Table S2 and S3) was observed. Altogether, these results are in line with the regulatory limits, thus confirming a satisfactory performance of method trueness and precision.

3.5. Complexity of Soil as a Matrix. Extractions of organic analytes from soil matrices pose an analytical challenge from the point of view of the diverse interaction occurring between soil and pollutants. Nonetheless, the overall recovery rates for all soil matrices and AFs in the present study were by around 11% higher than those presented by Starr and Selim,[8] despite using a supercritical fluid extraction approach. Strong interactions between AFs and soil organic matter were demonstrated by Schenzel et al.,[10] with log K_{OC} values ranging from 2.80 to 3.46. The authors explained that structural differences between the AFs were responsible for the different K_{OC} values. AFs with a double bond such as B1 and G1 resulted in a higher affinity for peat by ~0.45 log units compared to saturated forms (B2 and G2). Furthermore, van Rensburg et al.[11] observed strong interactions between AFs and the humic acid oxihumate with binding capacities of 7.4−11.9 mg AFB1 per g of oxihumate over a pH range of 3−7. Clay minerals constitute also effective sites for interactions with AFs.[4,5,12,13] Results from Goldberg and Angle[9] suggest that the sorption affinity of AFs to clay minerals may be higher than that to soil organic matter, as a relationship was found between the adsorption coefficient and clay content but not for organic carbon content. Kang et al.[47] postulated that electron-donor−acceptor interactions between the two electron-rich carbonyl groups $(C{=}O)_2$ in the coumarin structure of the AFs and electron-deficient or positively charged species located at the negatively charged surface of clay minerals (i.e., H^+ for illite and Ca^{2+} for smectite) are mainly responsible for the strong sorption of AFs to 2:1 clay minerals. However, the analytical method presented in this study was able to overcome these interactions in soils, resulting in suitable values in terms of recovery. The combination of MeCN/H_2O solvent extraction with ultrasonication was able to successfully extract AFs from soil matrices with clay contents up to 40.9% and organic carbon contents up to 1.99%. Ultrasonication has shown to significantly decrease the particle size of soil agglomerates[48] and clay minerals[49−51] and hence increase the surface area, resulting in a more intense contact with the extraction solvent. MeCN is a monopolar solvent that exhibits H-bond acceptor properties (solute H-bond basicity = 0.32)[52] but insignificant H-bond donor properties (solute H-bond acidity = 0.07)[52] and hence behaves similar to the carbonyl groups in the coumarin structure of the AFs. Thus, MeCN may competitively displace the AFs from H-bond-accepting sites of the cations, which are located on the negatively charged clay mineral surfaces. Madden and Stahr[6] used a solvent mixture of similar composition (MeCN/H_2O, 9:1), but only trace amounts could be recovered. This may be due to a missing ultrasonication step or an insufficient extraction time (4 min). Chloroform, one of the extractants tested by Mertz et al.,[4] is a monopolar solvent with insignificant H-bond acceptor properties (solute H-bond basicity = 0.02)[52] and therefore may not be able to compete with AFs for sorption sites. MeOH, the second extractant tested by Mertz et al.,[4] is a bipolar solvent with both H-bond donor (solute H-bond acidity = 0.43)[52] and acceptor properties (solute H-bond basicity = 0.47).[52] Hence, MeOH is also capable of interacting with itself, which may lower the ability to compete with AFs for sorption sites. In addition, the proton acceptor and donor sites are adjacent (within the OH group). Thus, the partial positive charge of hydrogen in the OH-group could hinder the attachment of MeOH to the positively charged cation layer. Angle and Wagner[5] and Goldberg and Angle[9] used acetone for extraction experiments, which is a monopolar solvent exhibiting H-bond acceptor properties (solute H-bond basicity = 0.49).[52] Therefore, acetone can be expected to compete for sorption sites to a similar extent as acetonitrile. While Angle and Wagner[5] were able to only recover 18% of the spiked amount, Goldberg and Angle[9] achieved recovery values of around 70%. This discrepancy may be explained by the fact that Goldberg and Angle[9] presaturated the soil with water before spiking with AFs. Hence, the interaction sites of the clay minerals may already be occupied by water molecules, lowering the affinity of AFs to clay minerals. It has already been shown that hydration of the soil prior to extraction and mixing organic solvents with small amounts of water weakens the interactions of analytes within the soil matrix and makes the pores in the soil more accessible to the extraction solvent.[53] In the case that only monopolar solvents with H-bond acceptor properties are able to successfully compete for sorption sites with AFs,

solvents such as alkenes, alkylaromatic compounds, ethers, ketones esters, and aldehydes could also be suitable candidates for the extraction of AFs from soils.

4. CONCLUSIONS

For the first time, a simple and reliable method is presented for the quantitative analysis of AFs in soil and food matrices at environmentally relevant concentrations using the same extraction procedure and chromatography, either by HPLC-FLD or LC–MS. Method validation according to the EuraChem guide[23] indicates the suitability of the method that is also in agreement with precision and recovery requirements of EC Regulation no. 401/2006[24] for AFB1, AFB2, AFG1, and AFG2 in four soils and five food matrices. Sensitivity allowed quantitative analysis even at trace levels (LOQ between 0.062–0.23 μg kg^{-1} for LC–MS and 0.035–0.231 μg kg^{-1} for the HPLC-FLD. As far as we know, this is the first solvent extraction method presented that achieves suitable and reproducible recovery rates for AFs in soil matrices (in particular in clayey soils) and the first method that does not require extract dilution or cleanup. The necessity for sample purification could be avoided since (i) matrix-matched calibration was capable of compensating matrix effects for LC–MS and (ii) interference peaks could be successfully separated using a weak elution program with a high water content for HPLC-FLD. Furthermore, since the matrix effect was negligible for HPLC-FLD, no matrix-matched calibration would be required and thus, solvent calibration would be sufficient. The absence of a purification step and the possibility to use HPLC-FLD significantly reduces the workload and costs. Therefore, the present method is of particular interest for routine analysis in countries in which levels of AFs may pose a health concern and continuous monitoring is needed in order to assess environmental contamination levels. This simple and rapid method offers also a possibility of capacity building since nonsophisticated analytical tools are needed. However, it remains to be clarified how SPE- or IAC-based purification methods perform in comparison with the presented method. For example, an additional SPE or IAC step could be used to concentrate the extracts, which may not be possible with the present method since a strong peak broadening of the interfering peaks could occur due to column overload. For other food/soil matrices, it may not be possible using the present method, and in this case, sample cleanup techniques such as IAC or SPE are advisable. Finally, the presented method opens up the possibility of reliably assessing the occurrence of AFs in the soil–plant system in agricultural areas. The insights gained from this could help in understanding the factors that lead to preharvest contamination and developing agricultural applications to reduce contamination in the field.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c01451.

Validation parameters, mean and relative standard deviation of aflatoxin recoveries, summary of statistic models, HPLC-FLD chromatograms, and solvent and matrix-matched calibration (PDF)

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Notes

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■ ABBREVIATIONS

AFs, aflatoxins; AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; AFG2, aflatoxin G2; AGC, automatic gain control; CEC, cation exchange capacity; C_{org}, organic carbon content; HPLC-FLD, high-performance liquid chromatography with fluorescence detection; LC–MS, liquid chromatography–mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MeCN, acetonitrile; MeOH, methanol; SSE, signal supression/enhancement; USE, ultrasonication-assisted solvent extraction

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Chapter 3

Aflatoxin Occurrence in Kenyan Maize Fields

The research in this chapter was carried out as part of the collaborative project, entitled "AflaZ: Zero Aflatoxin - A Multidisciplinary Collaboration between German and African Research Institutions". This field study is a joint project of the University of Kaiserslautern-Landau (RPTU), the Kenya Agricultural and Livestock Research Organization in Kabete (KALRO Kabete), and the Max Rubner Institute in Karlsruhe (MRI Karlsruhe).

Analysis of Aflatoxins in Soils: Insights Gained from a Maize Field Study in a Kenyan Hotspot Region

3.1 Introduction

Aflatoxins are toxic secondary metabolites produced by certain fungi of the anamorphic genus *Aspergillus*, which are commonly found in (sub)tropical soils where staple crops such as maize are grown (Mahuku et al., 2019). They can enter the food chain at any stage of production by fungal infestation, from pre-harvest to post-harvest, and pose a serious health risk to humans and animals (Winter and Pereg, 2019). In developing countries, with favorable (sub)tropical conditions for the growth and aflatoxin formation of these fungi and limited access to control measures, outbreaks of aflatoxin contamination are common and can lead to hundreds of deaths from acute aflatoxicosis. In addition, aflatoxin contamination causes significant economic losses to the agricultural and food processing industries (Winter and Pereg, 2019). In Kenya, maize is an essential crop that serves as both as a food and as an income source for the local population (Mahuku et al., 2019). Unfortunately, maize is highly susceptible to infection by aflatoxigenic fungi and contamination by AFs, thus contaminated maize presents a severe threat to the health of Kenyan consumers who rely on maize as their staple food with an average per capita consumption of 400 g per day (Lewis et al., 2005). Furthermore, more than 75% of maize in Kenya is produced by smallholder farmers for their own consumption, with the surplus being informally traded (Mahuku et al., 2019). As a result, the country has witnessed multiple outbreaks of acute aflatoxicosis since 2004, leading to nearly 500 acute illnesses and 200 deaths (Lewis et al., 2005). The economies of most tropical countries depend heavily on the export of agricultural products (Matumba et al., 2015). However, importing countries, especially those in the European Union, have imposed strict legal limits on aflatoxin levels, forcing large-scale farmers to commercialize corn within the acceptable limits to these countries, while selling highly contaminated maize on the informal market or consuming it locally (Matumba et al., 2015; Nji et al., 2022). As a result, the likelihood of the local population consuming contaminated food increases

(Nji et al., 2022; Udomkun et al., 2017), leading to additional economic costs such as disease costs (Meijer et al., 2021). This situation exacerbates the health and economic problems resulting from maize contamination with aflatoxins in Kenya. Therefore, implementation of mitigation strategies to reduce aflatoxin levels in maize is critical to meet regulatory requirements, avoid crop rejection and protect consumer's health.

The most important strategy to prevent pre-harvest aflatoxin infection and resulting aflatoxin contamination is through the use of suitable soil and crop management practices (Fouché et al., 2020; Verheecke et al., 2016). Different pre-harvest strategies have been proposed to reduce the incidence of aflatoxins in maize, including the use of biological control agents, crop rotation, intercropping, and less invasive soil cultivation practices. Studies have shown that the application of atoxigenic strains of *A. flavus* (Probst et al., 2011) or other atoxigenic mold fungi such as *Trichoderma harzianum* (Ren et al., 2022; Dania and Eze, 2020; Sivparsad and Laing, 2016) can outcompete aflatoxigenic strains, leading to reduced growth of the toxin producing fungi. The commercial product called "Aflasafe", based on atoxigenic *Aspergillus* strains, is available in the African market and is being used for field control of aflatoxigenic fungi (Migwi et al., 2020; Bandyopadhyay et al., 2016). Intercropping, the practice of growing multiple crops together, can also reduce the occurrence of phytopathogenic fungi by disrupting the fungus's spore dispersal patterns and suppressing pest insects (Trenbath, 1993; Langer et al., 2007). Pest insects can cause physical damage to crops and facilitate spore transport through wounds, thereby exacerbating fungal infection. Intercropping can help to alleviate this issue by creating a physical or chemical barrier (Trenbath, 1993; Langer et al., 2007). In context of maize cultivation, a "push–pull" intercropping technique has been demonstrated to be an effective method for controlling important maize pests such as several stemborers and the fall army worm (*Spodoptera frugiperda*) and reducing AFs prevalence in maize fields (Njeru et al., 2020). This technique involves intercropping maize, with insect-repellent forage legumes from the genus *Desmodium* and planting Napier grass (*Pennisetum purpureum*) around the field. The *Desmodium* legume releases semiochemicals that repel stemborer moths ("push") while simultaneously attracting them to the Napier grass ("pull"), which release attractive volatile organic compounds (Njeru et al., 2020;

Khan et al., 2000; Khan et al., 2011). However, the Napier grass does not support significant survival of emerging larvae, thereby preventing their destruction of the grass (Njeru et al., 2020; Khan et al., 2011). The push-pull system also effectively controls the fall army worm, an invasive pest that recently invaded Africa and attacks maize and other crops (Njeru et al., 2020; Khan et al., 2011). Conservation tillage is a practice that limits the disruption of the soil's structure, primarily through non-inversion techniques. By leaving over one-third of the soil surface covered by crop residues, conservation tillage promotes and sustains soil health while preventing soil degradation (Peigné et al., 2007). Conservation tillage offers several benefits in organic farming, such as decreased erosion, enhanced macroporosity on the soil surface due to an increased number of earthworms, higher microbial activity, and greater carbon storage (Busari et al., 2015). Additionally, it lowers nutrient run-off and leaching, minimizes fuel consumption, and speeds up tillage (Busari et al., 2015). Consequently, by improving soil health, conservation tillage may enhance plant health and lead to greater resistance against fungal pathogens. Conversely, if crop residues contaminated with aflatoxigenic fungi are left on the field for an extended period, there is a risk of soil re-contamination and subsequent crop contamination (Fouché et al., 2020; Accinelli et al., 2008; Angle, 1987). Since AFs are known to be toxic to certain soil microorganisms (Burmeister and Hesseltine, 1966; Arai et al., 1967), an increase in their presence in soil may affect the ecological balance of the soil microbiome and associated functions.

The effects of pre-harvest mitigation strategies have been studied mainly in terms of plant health and plant contamination by fungi and their toxins, but very little information is available on aflatoxin contamination and its toxicological consequences for the soil ecosystem. These strategies are reported to alter "aboveground" aflatoxin occurrence, but the consequences for "belowground" aflatoxin occurrence are literarily unknown. Consequently, the aim of the present study was to investigate whether agricultural practices change the concentration of AFs in field soil. These practices included push-pull intercropping, application of a suspension of *Trichoderma harzianum*, conservation tillage, and a control that followed conventional farming practices, as typically practiced by local small scale farmers. In the context of conservation

tillage, contaminated residues may remain on the soil surface, potentially acting as a source of AFs. Therefore, I hypothesize elevated AF levels in fields with conservation tillage and in particular at the top-soil layer. Furthermore, since the microbial and physico-chemical soil conditions in root space and inter-plant zone differ, I assume that there are differences in the production and transformation processes of AFs, which is reflected in differences in the levels of AFs. The farming practices "Trichoderma" and "push-pull" have been reported to decrease the presence of aflatoxigenic fungi, enhance the crop plants' resistance to fungal infestation and reduce aflatoxin prevalence in crops. Therefore I, hypothesize reduced AF levels in soils managed with these practices compared to those managed conventionally.

3.2 Material and Methods

3.2.1 Study Design and Field Treatments

The field study was conducted within the framework of the AflaZ project. The AflaZ project (Project AflaZ: Zero Aflatoxin) is an international and multidisciplinary research collaboration between German and African research institutions, aimed at reducing aflatoxin contamination in maize and milk in Kenya. The project focuses on the development of strategies to reduce the formation of aflatoxin by investigating the overall context of the problem, including plant protection, soil health, insect control, and molecular biological analyses, as well as knowledge transfer, networking and capacity building.

As part of the AflaZ project, a field study was carried out in a high-risk model region for aflatoxin contamination in Sub-Saharan Africa, namely maize fields in the Makueni region. The Makueni region was purposely selected based on previously reported outbreaks of aflatoxicosis (Lewis et al., 2005). The field experiments and sample collections were organized and executed by the Kenya Agricultural and Livestock Research Organization (KALRO), while local farmers carried out the maize planting according to the instructions provided by the institution. Over the course of three consecutive growing seasons, the effect of treatments on fungal and aflatoxin contamination in soil and maize grain was examined. The study involved 12 farmers

from each of three villages in Makueni County: Ukia, Kisau-Kiteta, and Nzaui-Kilili-Kalamba. Two different types of crops were used, DK 8031 maize and KAT B1 beans, as well as Duma 43 and KAT B1 beans during the long and short rain season. Maize was planted at a spacing of 75cm x 30cm with two seeds per hill and a row of beans spaced at 4cm between the maize rows. Conservational tillage was applied by spraying with Gramoxone® (25.4% active ingredient Paraquat, Syngenta, Basel, Switzerland) at the recommended field application rate after planting the maize

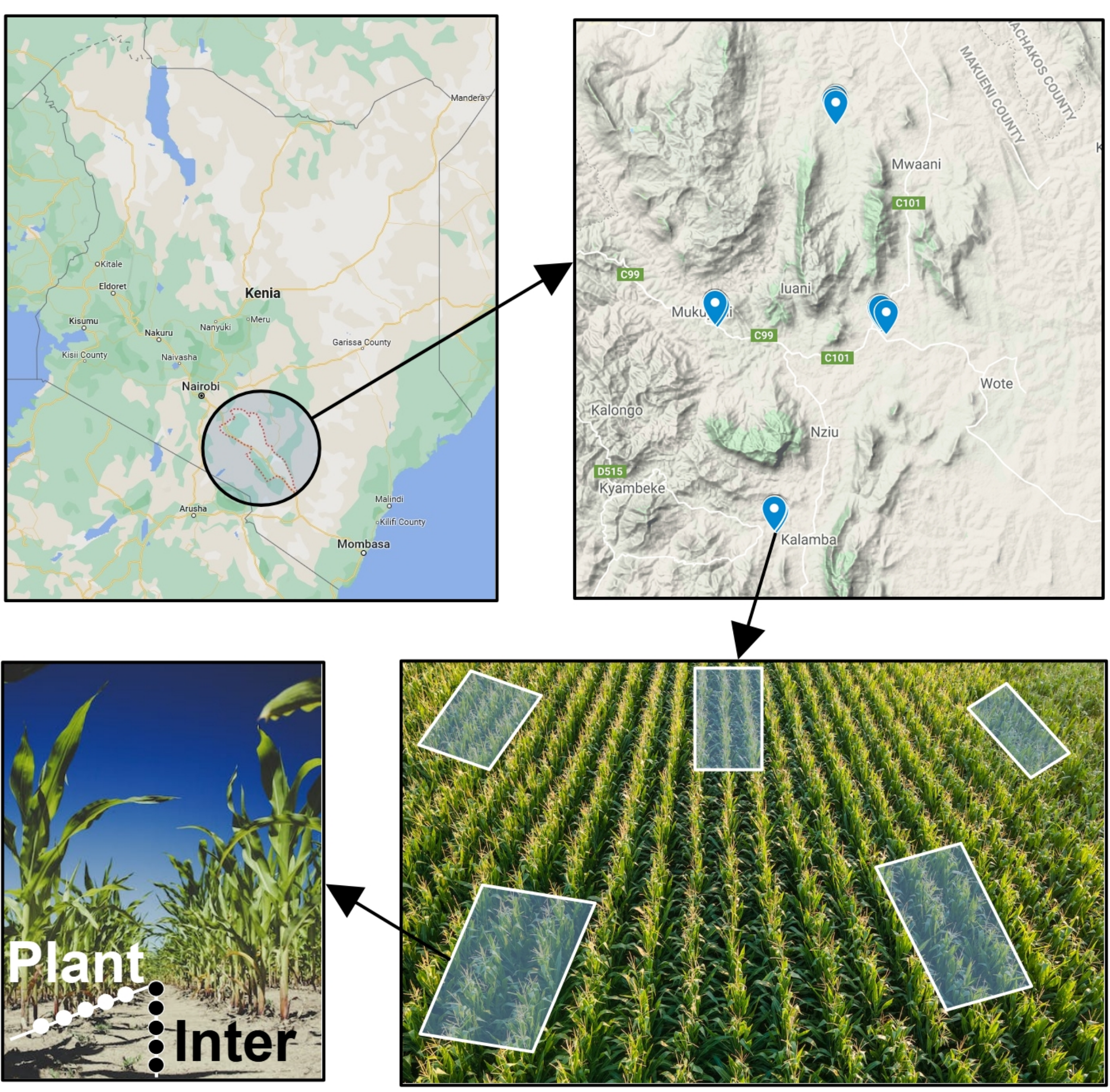

Figure 3.1: Study sites and sampling scheme for maize fields in the Makueni region (highlighted in top left map). 16 fields were selected within the Makueni county (markings in the top right map). Five clusters were designated on each field (bottom right). Each cluster was sampled on two positions at-"Plant" and "Inter"-row (represented by white and black dots, respectively) and two depths (top- and subsoil). Map data: ©2022 Google, Sanborn, USA.

and legumes, but before the two crops had germinated. For the remaining three treatments, hand weeding was done after planting maize intercropped with beans or desmodium depending on the plots. A total of 16 fields from 4 regional clusters, each farmed with one of the respective treatments, were sampled (Figure 3.1).

3.2.2 Soil Sampling Procedure

During the third season of maize cultivation in August 2020, soil sampling was conducted for the present study. Following a Standard Operating Procedure (SOP) developed at the University of Kaiserslautern–Landau (RPTU), the sampling was conducted with the support of KALRO (SOP available in Chapter 8.3). Five sub-plots of approximately 500 m^2 were defined on each field, with sampling points evenly distributed within each sub-plot. Soil samples were collected at two depths (0-15 and 15-30 cm) and two positions (between plants and inter-row) to detect potential concentration differences. Ten samples were taken for each position-depth combination, resulting in ten individual samples per sub-plot. Sampling was conducted by pushing an augur vertically into the soil to a depth of approximately 35-40 cm and separating the soil core into topsoil and subsoil fractions. Individual samples were pooled to create one sample for each position-depth combination within a sub-plot. The sampling design is visualized in Figure 3.1. Sampling was completed by August 31, 2020, and the samples were stored at 4°C in the dark until shipment. Soil samples were shipped to Germany on September 16th, 2020 for aflatoxin analyses. Upon arrival in Germany on November 16th, 2020, the samples were immediately quarantined at room temperature for 3 weeks. Subsequently, they were stored at -20°C until further processing.

3.2.3 Aflatoxin Analysis

Soil samples were passed through a 2 mm stainless steel sieve and analyzed for AFB1, AFB2, AFG1 and AFG2 according to Albert et al. (2021). Briefly, soil samples were extracted with MeCN:H_2O at a soil:solvent ratio of 1 g : 3 mL by orbital shaking and ultrasonication treatment and analyzed via both, high performance

liquid chromatography with mass spectrometry (LC-MS) and fluorescence detection (HPLC-FLD). The presence of interference peaks and matrix effects was tested by comparing a matrix-matched and solvent calibration (Albert et al., 2021). A matrix blank solution was prepared by combining equal volumes of all extracted samples.

3.3 Results and Discussion

3.3.1 Absence of aflatoxins in the soil samples

No aflatoxins were detected in any of the 320 soil samples. The chromatograms of both HPLC-FLD and LC-MS showed no interferences in the time window of the respective aflatoxins in both, the samples and the matrix blank solution. As a result, the initial research question concerning the influence of farming practices on the presence of aflatoxins in field soils remained unresolved. Nevertheless, this outcome has raised supplementary questions regarding the absence of aflatoxins in the examined soils. There are several possible explanations for the non-detectability of aflatoxins: (1) absence of any aflatoxigenic fungal strain in the fields studied; (2) Insufficient sampling strategy to detect aflatoxins in the soil; (3) (Photo)chemical and microbial degradation of aflatoxins in the field and/or during sample transport to Germany. These potential causes will be evaluated for their plausibility in the subsequent sections.

3.3.2 Aflatoxigenic Strains in the Field Soil

It is reported that there are temporal dynamics, both within and between years, regarding the occurrence of toxigenic fungal strains and mycotoxins in crop plants and debris (Abbas et al., 2008; Orum et al., 1997; Ching'anda et al., 2022). These dynamics can be due to various reasons such as climatic conditions, previous agricultural practices and the stage of development of the crop (Ching'anda et al., 2022; Dutta and Das, 2001; Jaime-Garcia and Cotty, 2010). It is therefore conceivable that the field sampling fell into a time window with a low occurrence of toxigenic strains and/or reduced aflatoxin production. However, this is contradicted by the fact that toxigenic strains

were found in the same soil samples by another team of researchers from the MRI (presented at the AflaZ congress on October 13th, 2022 in Nairobi, Kenya). The ability of fungal strains of *A. minisclerotigenes* and *A. flavus* isolated from soils, to produce AFs was confirmed through the detection of AFs in extracts from cultivated fungal strains using thin-layer chromatography with fluorescence detection. Additionally, findings presented by the KARLO-Kabete Institute at the AflaZ congress on October 13th, 2022 (Nairobi, Kenya), indicated the presence of toxigenic strains in the fields, as demonstrated by the detection of AFs in grain samples. Overall, the absence of aflatoxigenic strains in the fields seems rather implausible as an explanation for the non-detectability of AFs in the soil samples.

3.3.3 Sampling Strategy to Detect Aflatoxins in the Soil

Agricultural soils exhibit inherent heterogeneity, both spatially across the field and vertically within the soil profile, leading to potential variations in mycotoxin levels even within a small area. Certain plant debris, particularly grain-rich material, is often heavily colonized by toxigenic *Aspergillus* fungi, making them potential "hot spots" for aflatoxin contamination, with concentrations reaching up to 10^2 µg kg^{-1} (Accinelli et al., 2008). Therefore, one possible explanation for the absence of detectable AFs in soil samples could be an inadequate sampling procedure that failed to capture these localized "hot spot" areas within the soil. However, this assumption is contradicted by the successful application of the same sampling strategy in maize field soils in Germany to detect *Fusarium* toxins including nivalenol and deoxynivalenol (Kenngott et al., 2022). Therefore, this explanation also appears implausible, particularly considering that soil extracts from the Kenyan soils were also subjected to the same analytical procedure described by Kenngott et al. (2022) and tested negative for the presence of *Fusarium* toxins, including nivalenol, deoxynivalenol, 15-acetyl-deoxynivalenol, and zearalenone.

3.3.4 Aflatoxin Degradation

Soil sampling was conducted during the harvest season, which fell into the dry season with low moisture and high temperature conditions. Farmers leave the maize plants in the field for several weeks during this period in order to decrease the moisture content of the kernels before harvesting. This initial drying in the field effectively reduces moisture content and prepares the crop for the subsequent drying processes required for storage and sale. The plants undergo senescence, and the leaves begin to wilt, consequently reducing leaf coverage and allowing more sunlight to reach the ground. As a result, aflatoxin-contaminated material on the soil surface and in the topsoil layer could be exposed to UV light. It is well established that AFs are highly susceptible to photolytic degradation, with a half-life ranging from a few hours to days under direct irradiation (Diao et al., 2015). This suggests that photolytic degradation of aflatoxins may have occurred in the field shortly before sampling, which could explain the absence of aflatoxins in the collected samples.

In addition, a period of 2.5 months passed between sample collection and their arrival in Germany. While the samples were kept refrigerated at 4°C until being shipped, they were not refrigerated during the 2-month transport period. This extended duration may have led to the degradation of AFs. Furthermore, the samples were subjected to extremely dry conditions during the dry season, characterized by a lack of rainfall and high temperatures, which would have resulted in significantly reduced microbial activity. However, it is also possible that abiotic degradation occurred in the absence of light. It is important to note that the prevailing consensus is that abiotic degradation of aflatoxins does not play a major role in the soil environment. However, the experimental evidence is very limited- In this regard, only two studies have conducted degradation experiments under (near) sterile conditions, and their findings indicated minimal degradation of AFB1 (Accinelli et al., 2008; Starr et al., 2017). Considering these factors, the likelihood of abiotic degradation during transport appears less plausible.

Microbial degradation is widely recognized as a significant process contributing

to the removal of aflatoxins in soil (Fouché et al., 2020). Previous studies have reported almost complete dissipation of AFB1 at concentrations ranging from 10^0 to 10^4 µg kg^{-1} within a week in soil (Angle and Wagner, 1980; Angle, 1986; Accinelli et al., 2008). However, considering the arid conditions prevailing during the harvest season in the field, it is expected that microbial activity will be severely limited. Additionally, the low moisture content observed in the soil samples suggests that microbial degradation during shipment is unlikely. During the transitional phase between seed ripening and senescence, nutrient, moisture and temperature conditions are heavily changing towards unfavorable conditions i.e. drought, heat and reduced root exsudation (Zhalnina et al., 2018; Cotta et al., 2012). However, large amounts of carbon and nitrogen are released by dead roots and it was shown that soils can be hotspots of microbial activity at this stage (Spohn and Kuzyakov, 2014). Thus, during this phase, any existing AFs in the soil could still potentially undergo microbial degradation. Consequently, microbial degradation during the transition to the senescence phase could have contributed to a reduction in soil contamination before and at early stages of the harvest season, even if minimal aflatoxin degradation is expected during later stages of the harvest season with very dry soil conditions.

3.4 Conclusion and Future Perspectives

Suitable soil and crop management practices are considered the major means of preventing pre-harvest aflatoxigenic fungal infestation and aflatoxin contamination and thus ensure soil health and productivity. The present study was therefore designed to investigate how different farming practices affect the occurrence and distribution of aflatoxins in the soils of maize fields. However, the research question could not be addressed due to the absence of aflatoxins in any of the soil samples. The absence of AFs in the soil during the whole period of maize cultivation seems unlikely in view of the proven presence of toxigenic strains in the samples. Since the mycotoxin producing fungi were identified in the soil samples, this may have the potential of a production *in situ* in the early stages of plant growth (i.e. when soil moisture recovers) with the risk of a translocation from soil to plant. Moreover, methodological

shortcomings in the sampling strategy seem improbable, as previous studies have detected mycotoxins in maize field soils using the same sampling strategy. Rather, a (photo)chemical or microbial degradation of the aflatoxins up to non-detectability in the field, during sample storage or the extended period of sample transport may have occurred. To gain a comprehensive understanding of the persistence and environmental behavior of AFs in soil, I recommend conducting microcosm experiments under controlled conditions to evaluate the likelihood, significance, and mechanisms of the various degradation processes. Furthermore, for future studies on the impact of farming practices on pre-harvest soil contamination with aflatoxins, I propose a temporally close-meshed sampling over the entire course of the maize cultivation period to understand the temporal dynamics in aflatoxin occurrence. In addition, I emphasize the importance of conducting prompt analyses of soil samples subsequent to sampling to minimize potential degradation during transportation and storage, highlighting the necessity for capacity building and close collaboration with local analytical partners to establish analytical capabilities in close proximity to the fields.

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Chapter 4

Soil Environmental Fate of Aflatoxins

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Kinetics of microbial and photochemical degradation of aflatoxin B1 in a sandy loam and clay soil

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In a 28-days experiment, we investigated the dissipation of aflatoxin B1 (AFB1) (0.5–500 μg kg^{-1}) by microbial (MD) and photodegradation (PD) in two contrasting soils (sandy loam and clay). Sterile incubation in darkness served as control (C). AFB1 was degraded in all scenarios according to simple first-order kinetics with 50% dissipation times of 20–32 (PD), 19–48 (MD), and 56–65 days (C), respectively. Dissipation rates were significantly lower ($p < 0.001$) in the clay soil than in the sandy loam soil, likely due to photoquenching and strong binding of AFB1 by clay minerals and humic substances. In the sandy loam, dissipation rate of MD decreased in function of initial AFB1 concentration, probably due to toxic effects on degrading microbes. In contrast, in the clay soil the dissipation rate increased with increasing concentration up to 250 μg kg^{-1}, followed by a sharp decrease at 500 μg kg^{-1}, indicating an effect of soil texture on the bioavailability of AFB1 to soil microbes. AFB2a was identified as a transformation product in all scenarios. These results confirm the function of soil for AFB1 degradation, which is modulated by abiotic and biotic processes, soil characteristics and initial AFB1 concentration.

Aflatoxins (AFs) are toxic secondary metabolites produced by several species of the fungal genus *Aspergillus*. The occurrence of AFs in food and feed commodities has been associated with serious health consequences for humans and animals[1] and substantial economic losses for agriculture[2] and livestock[3]. Soil is considered a natural habitat for filamentous fungi including aflatoxigenic strains and serves as a reservoir for primary inoculum for the infection of plants[4]. AFs can be synthesized in situ or introduced into the soil when contaminated plant residues or food from storage systems are buried in the soil for natural degradation[5,6]. The presence of AFs in agricultural soils has been reported, with concentrations ranging from 10^{-2} to 10^{1} μg kg^{-1}[5]. Further, the occurrence of AFs has the potential to alter the ecological balance in soil[6,7], namely the structure and functions of microbial communities. Specifically, AFs can affect soil bacteria, fungi, and actinomycetes[8], thus impairing associated soil biogeochemical processes. In the context of assessing the environmental relevance of a toxic pollutant, the question of its persistence in the environment in which it occurs, arises since the rate of dissipation largely determines the duration and intensity of ecotoxicological effects. Dissipation processes in soil are driven by microbial, physical and chemical factors. Since the conditions of the respective degradation processes are different, the rate of dissipation and the resulting transformation products may also vary. The resulting transformation products may be more toxic and persistent than parent compounds[9], thus investigation on metabolites are essential. Soil has been largely overlooked as a potential sink of AFs and as a matrix in which transformation reactions take place. To understand the environmental relevance of AFs in soil, investigations on the rate at which AFs dissipates from soil and the processes that lead to their dissipation are imperative.

Microbial and enzymatic degradation of AFs has been summarized by Wu et al.[10] and Verheecke et al.[11]. Most studies have so far focused on the potential application of such approaches for the detoxification of food and feed commodities. Such studies were performed in vitro using bioreactors, liquid and agar cultures, or matrix specific media and carried out with single species or their isolated enzymes which do not originate from the environment in which aflatoxigenic fungi and their toxins normally occur. These include wood decaying fungi[12,13], microorganisms isolated from soils that are highly polluted with persistent organic pollutants[14,15], microorganisms that are used in the food processing industry[16] and microorganisms isolated from the digestive tract[17,18]. Therefore, the reported almost complete degradations of AFB1 within a few hours to days under in vitro conditions may

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be optimistically high compared to natural degradation in soil. Dissipation of AFB1 in soil was observed at concentrations of 10−50 mg kg^{-1} with nondetectability in less than 6 days[19,20] and at 10 µg kg^{-1} where 50% of the initial mass dissipated (DT_{50}) in less than 5 days[5]. After 112 day of incubation, 1.4–14% of the applied AFB1 was mineralized[19,20]. The mineralization rate was about one sixth slower in the silty clay loam as compared to the silt loam soil. Hence, it was concluded that the clay content and organic content of the soil had a negative effect on the degradation and mineralization rate, which was explained by a lower bioavailability due to sorption of aflatoxins in the corresponding soil compartments[20]. AFs are known to have a medium strong sorption affinity for organic carbon[21,22] and a particularly strong sorption affinity for clay minerals[19,20,23–26], thus reducing the bioavailability for the degrading microbes[25]. In this context, Goldberg and Angle[26] have shown that AFB1 adsorption coefficient was about five times higher in a less humic (0.6% C_{org}) silty clay loam soil (37.8% clay) compared to a much more humic (2.9% C_{org}) silt loam soil (33.6% clay). Further, reduced mineralization of AFB1 in a silt loam soil fortified with 50 mg kg^{-1} AFB1 compared to the same soil enriched in 10 mg kg^{-1} AFB1 was observed[20], indicating an effect of initial AFB1 concentration on the AFB1 degradation rate. Interestingly, the same group[8] observed that the initial AFB1 concentration was related to the extent of the ecotoxicological effects observed with a continuous decrease of viable population of fungi, bacteria and actinomycetes in a agar media with 1, 100, 10,000 µg AFB1 L^{-1}. At the highest AFB1 level, the number of viable fungi, bacteria and actinomycetes was reduced by 38–34% compared to the control in the agar media. A similar situation was observed in AFB1 fortified soils where the effects started 2 weeks after AFB1 application and persisted for nearly 6 weeks[8]. When metabolites were identified using thin layer chromatography, the major metabolites detected were AFB2 and to a lesser extent AFG2 and AFG1[19,20]. However, Starr et al.[27] found only AFB2a as a single transformation product in an aqueous-soil environment product using HPLC-UV and HPLC-MS for analysis. The authors remarked that the use of thin-layer chromatography may have led to misidentification of metabolites.

In AF hot-spot regions, harvest season often coincide with dry periods[4,28], conditions that are also observed in the soil. As a result of soil dryness, reduced microbial activity and AFs decomposition is likely. Thus, AFs may undergo physicochemical rather than microbial degradation during this season. To date, numerous physical and chemical conditions are known to detoxify aflatoxins in food matrices as summarized by Pankaj et al.[29] and Guo et al.[30] including: UV light, organic acids, ammonia, formaldehyde, ozone, sulfites, hydroxides and hypochlorites. These approaches has not been so far investigated in soils, although soils are exposed to UV irradiation in sunny and dry periods. Further, agricultural practices (e.g. fertilization, liming, tillage), plant root exsudation and biochemical transformation reactions can favor the formation of reactive substances in the soil such as organic acids and sulfites, that may initiate chemical degradation of AFs. Another aspect to be considered in degradation process in the soil is the texture and composition, such as clay minerals and humic substances, as these compartments can protect chemicals from degradation reactions due to their steric rearrangement into adsorption sites[31] or can catalyze physicochemical degradation processes on their surfaces[27,32–35]. So far only two studies investigated the AFB1 degradation under (almost) abiotic conditions. Accinelli et al.[5] observed no degradation in an autoclaved soil incubated in the dark. Hence, the authors concluded that AFB1 degradation in soil is mainly driven by microbial processes. Starr et al.[27] observed no AFB1 dissipation in a dry silty loam soil after 60 days of incubation (in dark). Although the soil was not sterilized prior to incubation, microbial activity and thus biodegradation was considered insignificant because of insufficient soil moisture.

Soil is the natural habitat of aflatoxin-producing fungi and a disposal medium for AF contaminated plant residues. However, the processes underlying AFB1 degradation in soil and how these relate to available AFB1 concentration and physicochemical soil properties have not yet been systematically investigated. In addition, only microbial degradation has been studied as a mechanism of aflatoxin decomposition in soil, although aflatoxins in this system are exposed to other reactive abiotic conditions such as sunlight or chemical reagents. Therefore, the aim of this study was to elucidate the dissipation rate of AFB1 in two different soils (sandy loam and clay soil) under abiotic and biotic conditions. For this purpose, soils were amended with 50 µg kg^{-1} AFB1 and subjected to microbial degradation (MD) and UV light induced photodegradation (PD). Sterile soils amended with 50 µg kg^{-1} AFB1 and incubated in dark served as control. In addition, it was examined whether increasing initial concentrations of AFB1 (0.5–500 µg kg^{-1}) have an effect on the dissipation rate of AFB1 in soils subjected to MD. The samples were further analyzed for the formation of the previously described metabolites in soil matrices, i.e. AFB2, AFB2a, AFG1 and AFG2. Since clay minerals and humic substances can strongly bind AFs and attenuate light, we assume that (i) AFB1 is less available to soil microorganisms, enzymes and UV light in the more humic and clayey soil resulting in a reduced AFB1 dissipation rate. Because of the potential toxic effect of AFB1 on soil microbes, we expect (ii) a negative relationship between AFB1 dissipation rate and AFB1 fortification level.

Methods

Chemicals and reagents. Ultrapure water was used throughout all work (Milli-Q-water purification system, 18.2 M Ωcm^{-1}, EASYpure II, Millipore Bedford, MA). Acetonitrile (MeCN) and methanol (MeOH) used for extraction, reconstitution, chromatography and preparation of standards were of HPLC grade (Carl Roth, Karlsruhe, Germany). A standard mixture solution with certified concentrations of 20 mg L^{-1} each for AFB1, AFB2, AFG1, and AFG2 dissolved in MeCN (Sigma-Aldrich, St. Louis, USA) was used for preparation of external calibration standards. A stock solution containing 500 mg L^{-1} AFB1 was prepared by dissolving 10 mg crystalline AFB1 (from *Aspergillus flavus*, by Sigma-Aldrich, St. Louis, USA) in 20 mL MeCN which was then used for sample fortification. The concentration of the fortification standard was not significantly different from the nominal concentration of 500 mg L^{-1} (see SI-2 Quality criteria and pretests). A qualitative AFB2a standard was prepared as described by Rushing et al.[36]. Briefly, AFB1 (2.5 mg L^{-1}) was dissolved in 1 M citric acid solution (Carl Roth, Karlsruhe, Germany) to achieve a nominal concentration of 500 µg L^{-1}. This AFB1 solution was allowed to react for 72 h to form AFB2a. The AFB2a standard was then diluted to 5 µg L^{-1} with ACN and was

Property	R01A	L6S
Soil type	Sandy loam	Clay
Sand (%)	70.5	23.2
Silt (%)	26.1	35.5
Clay (%)	3.4	41.2
C_{org} (%)	0.9	1.7
WHC (%)	29.3	42.4
pH (0.01 M $CaCl_2$)	5.4	7.3
C_{mic} (mg kg^{-1})	95 ± 15	267 ± 8
SIR (mg CO_2-C kg^{-1} h^{-1})	3.8 ± 0.9	11.1 ± 2.3
BR (mg CO_2-C kg^{-1} h^{-1})	1.8 ± 0.3	3.7 ± 0.6

Table 1. Physicochemical and microbial (mean ± standard deviation, n=3) properties of the tested soils.

used for identification of AFB2a in sample extracts from the degradation experiments. All solutions were stored in the dark at −20 °C until analysis.

Soil characteristics. The degradation experiments were carried out using two soils. The sandy loam soil "R01A" ("RefeSol 01-A", Fraunhofer IME, Schmallenberg, Germany) and clay soil "L6S" ("LUFA 6S", LUFA, Speyer, Germany), both served as reference soils from organically managed arable areas (Table 1). Soils were purchased in field-fresh state and conditioned to meet the requirements of OECD 307[37] (see SI-2 Quality criteria and pre-tests), which was developed to evaluate the rate of transformation of a test substance, and the nature and rates of formation and decline of transformation products. A detailed description of the soil sampling and preparation is found in the supplementary information (see SI-2 Quality criteria and pretests). The soils correspond to the upper soil layer i.e. at 0–20 cm (L6S) and 0–25 cm (R01A) and were homogenized, 2 mm-sieved (stainless steel) and stored at 4 °C for less than 1 month. These soils were selected to cover a wide range of physicochemical and microbial properties, which are expected to have an influence on the dissipation of AFB1 i.e. organic carbon content, pH, soil texture, microbial biomass and activity (Table 1). The soil organic carbon and clay mineral contents, as reflected in soil texture (clay content), are of particular interest as these soil fractions represent sorption sites for AFs[23,25,38] as well as may attenuate the UV light[39]. Basal respiration (BR) and glucose-induced respiration (substrate induced respiration, SIR) of the soil were determined using the MicroResp setup[40] according to Schirmel et al.[41]. BR is the measured soil respiration after addition of water and represents a measure of the respiratory turnover of predominantly native carbon at steady state[42]. Initial soil respiration after addition of a readily available carbon source such as glucose (SIR) is proportional to the mass of metabolically active organisms and therefore serves as a bioindicator of active microbial biomass[43,44]. Total microbial biomass carbon (C_{mic}), which includes both the metabolically active and dormant fractions of the soil microbiome was determined using the chloroform fumigation extraction method[45]. Bulk soil was moisture adjusted to 40% water holding capacity and preincubated in dark at 20 °C for 1 week prior degradation experiments to reestablish equilibrium of microbial metabolism[37].

Degradation experiments. Microbial degradation experiments were carried out at four fortification levels with 0.5, 5, 50, 250 and 500 $\mu g\,kg^{-1}$ and a blank free of AFB1. Soils were fortified using acid washed quartz sand coated with AFB1 as carrier. Quartz sand was coated with AFB1 using a fortification standard containing 500 mg L^{-1} AFB1 dissolved in MeCN. MeCN was used instead of MeOH as a carrier solvent for sample fortification to prevent formation of artifactual methoxy aflatoxin species[27]. The solvent was allowed to evaporate for 1h before the fortified sand was added to the soil in order to avoid potential effects of the solvent carrier on soil microorganisms. A sand application rate of 1% was chosen according to the OECD[46]. The blank soil was prepared using the same procedure, but with MeCN. Fortified soil aliquots of 100 g were incubated in 200 mL polypropylene screw cap beakers in triplicate. To maintain aerobic conditions while minimizing water loss through evaporation, a filter was inserted into the screw cap by drilling a 1 cm hole into which polyester filter floss (Symec, JBL, Neuhofen, Germany) was placed.

Photodegradation experiments were carried out with 10 g (dry weight) aliquots of preincubated soils in 70 mL screw cap incubation glass jars. The incubation vessels had a base area of 24.5 cm^2 resulting in a uniformly spread soil layer of approximately 3.5 mm thickness. This thickness was sufficient for UV light to penetrate the soil layer. The jars were equipped with a septum for sterile injections and a 2 mm wide vent sealed with two layers of surgical tape (Micropore, 3M, Neuss, Germany) to allow gas exchange while preventing passage of microbial contaminants. Filled vessels were sterilized by autoclaving the soil for 30 min at 121 °C, followed by a second autoclavation run after 2 days in order to prevent potential recolonization by intact spores. Sterility was verified by absence of colony forming units by spreading sterilized soil on surface of sterile agar medium (15 $g\,L^{-1}$ agar, 5 $g\,L^{-1}$ peptone, 2.5 $g\,L^{-1}$ yeast extract, 1 $g\,L^{-1}$ glucose, pH 7.0, Carl-Roth, Karlsruhe, Germany). Soils were fortified by injecting 200 µL of of diluted AFB1 fortification solution (2.5 $g\,L^{-1}$ in MeCN) using a glass syringe equipped with a sterile filter (PET, 0.2 μm) into the incubation vessels through the septum to obtain a AFB1 soil concentration of 50 $\mu g\,kg^{-1}$. Potential AFB1 extraction losses due to adsorption to the glass material was

excluded (see SI-2 Quality criteria and pretests). Soil samples were incubated under UV irradiation from below with a UV fluorescent tube (40W, CLEO Performance N, Philips, Amsterdam, Netherlands). The UV irradiation received by the soil after absorption losses by the glass material had an intensity of 9.1 W m^{-2} UVA and 0.03 W m^{-2} UVB. Sterilized and fortified soil incubated in the dark served as control.

Evaporated water (checked gravimetrically) was replenished weekly by sterile injection of ultrapure water. The homogeneous distribution of AFB1 in the fortified soils was evaluated by spike recoveries at day 0 (see SI-2 Quality criteria and pretests, see Table SI-2). All incubation vessels were incubated at 20 °C and triplicate samples were removed and analyzed at 0, 1, 3, 8, 15, 22 and 28 days after fortification.

Aflatoxin extraction and analysis. Aflatoxins, namely AFB1, AFB2, AFG1 and AFG2, in the soil samples were extracted with MeCN:H2O (84 : 16, v + v) and analyzed via high performance liquid chromatography with fluorescence detection (HPLC-FLD), according to Albert et al.[38]. AFB2a was analyzed using the same method with excitation and emission wavelength of the fluorescence detector set to 365 and 455 nm. The retention time of AFB2a was determined by injection of the qualitative AFB2a standard (5 µg L^{-1}). All aflatoxins were quantified by external solvent calibration in the range of 0.05–10 µg L^{-1}. During photochemical post-column derivatization, AFB1 is completely converted to AFB2a by conversion of the double bond of the dihydrofuran moiety into hemiacetal derivatives[47]. This allows quantification of AFB2a peaks with the same external solvent calibration as AFB1.

AFB1, AFB2, AFG1, AFG2 and AFB2a were further confirmed using liquid chromatography-high resolution accurate mass spectrometry (LC-HRMS). Retention time and spectra for AFB2a were determined by injection of the qualitative AFB2a standard (5 µg L^{-1}). Target analysis was performed for the [M + H]+ adducts with ionic masses at 313.0715, 315.0860, 329.0650, 331.0800, and 331.0799 m/z for AFB1, AFB2, AFG1,AFG2, and AFB2a respectively. In addition, the corresponding $[M + NH_4]+$ adducts were continuously monitored to confirm the positive findings. The m/z of the $[M + NH_4]+$ adducts were 330.0962, 332.1132, 351.0467, 353.0631, and 353.0624 for AFB1, AFB2, AFG1, AFG2, and AFB2a, respectively. Example chromatograms and spectra can be found in the Supplementary Information (see SI-3 Chromatographic data, Figs. SI-1 and SI-2).

Data analysis. Data processing and statistical analyses were performed using R (version 4.0.3, R Core Team). Data manipulation, tidying and visualization was done using the the "tidyverse" package (available from https://doi.org/ggddkj)[48]. For all linear models (i.e. calibration, multiple regression and ANOVA models) the assumption of homoscedasticity was checked via scale-location-plots (square root of standardized residuals versus predicted values)[49] and the normality assumption was assessed via quantile-quantile plots[49]. Outliers were detected using the boxplot method[49]. Extreme points were defined as values above the third quartile + 3x interquartile range or values below the first quartile - 3x interquartile range. Test results were considered as significant when $p < 0.05$ and as marginally significant (trend of significance) when $p < 0.1$.

One sample t-test was conducted to evaluate significant differences between the measured AFB1 concentration in the fortification standard and the nominal concentration (see SI-2 Quality criteria and pretests). To check whether the AFB1 concentrations of the glass adsorption test (see SI-2 Quality criteria and pretests) differ between day 0 and day 8, a two sample t-test was performed.

AFB1 dissipation kinetics were assessed by fitting single first order kinetcs (SFO) to data using the Levenberg-Marquardt type fitting algorithm[50] with the command "nlsLM" (package "minpack.lm"[51]). SFO rate equations were fitted to the AFB1 concentrations changing with incubation time.

$$c = c_0 \cdot e^{-k_{SFO} \cdot t} \tag{1}$$

c_0 is the initial AFB1 at time t = 0 (d) and c is the AFB1 concentration at given time t (d) and k_{SFO} (d^{-1}) is the single first order dissipation rate. The resulting regression models were evaluated for their goodness of fit via visual inspection and Efron's pseudo coefficient of determination (R^2)[52]. According to the OECD307 guideline[37] SFO kinetics are favored over other kinetic models unless coefficient of determination $R^2 < 0.7$. All models fullfilled these requirements, except for 1 model (L6S, c = 0.5 µg kg^{-1}, MD, $R^2 = 0.593$). The insufficient fit of this model was due to an outlier at t = 3. The removal of this outlier before model fitting resulted in a R^2 of 0.765. In addition, there was an outlier in the nonsterile incubated L6S soil contaminated with 250 µg kg^{-1} on day 3, where the concentration was higher than the corresponding measurement on day 0. This outlier was also removed prior kinetic modeling. AFB1 dissipation kinetics were visualized by plotting normalized AFB1 concentrations c/c_0 against incubation time t which allowed comparison between different incubation conditions and fortification levels. To estimate the rate of AFB1 dissipation under each incubation condition, SFO kinetics were used to determine 50% dissipation times (DT_{50}). These values indicate the time t (d) within which the concentration of the test substance is reduced by 50%.

$$DT_{50} = \frac{ln2}{k_{SFO}} \tag{2}$$

All data used for kinetic modelling can be found in the supplementary information (see SI-1 Raw data for kinetic modelling of AFB1 dissipation, Table SI-1). The processes involved in the dissipation of AFB1 in the soils under the different incubation conditions were investigated with mass balance analysis. The respective fractions, i.e. extractable AFB1, extractable metabolites and non-quantifiable residues, were determined and expressed as a percentage of the initially applied amount of AFB1. The non-quantifiable fraction represents the initially applied amount of AFB1 minus the extractable amount of AFB1 and the metabolite AFB2a. This fraction represents a sum of numerous processes contributing to dissipation such as the formation of bound residues,

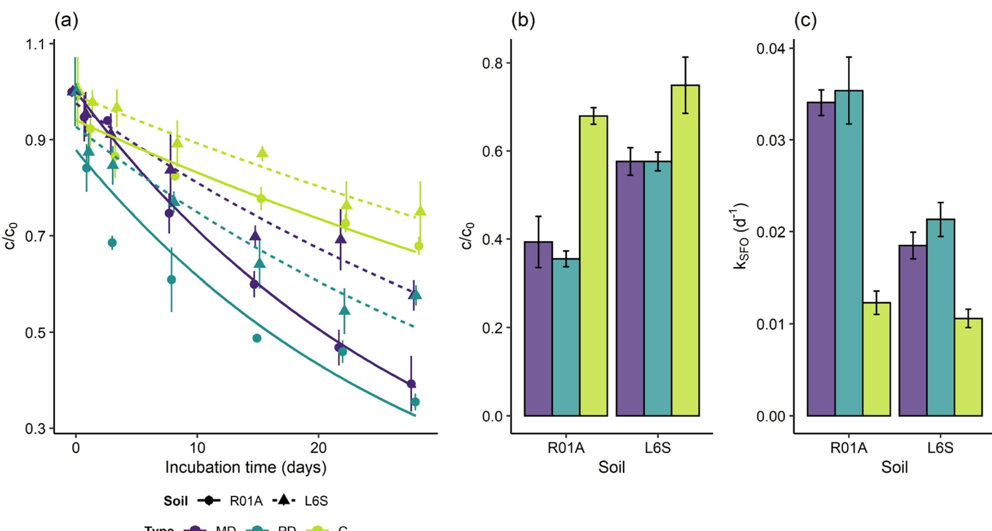

Figure 1. AFB1 dissipation for the sandy loam soil “R01A” (solid lines + points) and clay soil “L6S” (dashed lines + triangles) both fortified with 50 µg kg^{-1} AFB1 and subjected to microbial degradation “MD” (purple), photodegradation “PD” (dark cyan) and the sterile control in darkness “C” (light green). Curves showing single first order kinetic model fitted to data (**a**), normalized AFB1 concentration c/c_0 at the end of 28-days incubation (**b**) and single first order dissipation rate constants (**c**). Results are presented as mean ± standard deviation (n=3).

incorporation of AFB1 carbon into microbial biomass carbon, mineralization, volatilization and transformation (e.g. into other metabolites).

The dissipation kinetics of AFB1 were tested (i) between the different incubation conditions at the same AFB1 fortification level (50 µg kg^{-1}) considering the soil type (see SI-4 Statistical analyses, Tables SI-3, SI-4 and SI-5) and (ii) between the different AFB1 fortification levels at the microbial degradation scenario considering the soil type (see SI-4 Statistical analyses, Tables SI-6, SI-7 and SI-8). The effects of (i) the predictors degradation conditions (“Type”; factor with the three levels “C”, “MD” and “PD”) and soil type (“Soil”; factor with two levels “L6S” and “R01A”) and their interaction was tested using two-way ANOVA model. The effect of (ii) the predictors AFB1 fortification level (“Level”; numeric with the five levels “0.5”, “5”, “50”, “250” and “500”) and soil type (“Soil”; factor with two levels “L6S” and “R01A”) and their interaction on the AFB1 c/c_0 ratio at the end of incubation (day 28) was tested using a multiple regression model. In the case of a significant two-way interaction, post-hoc tests were performed to analyze the effect of the first predictor on the response variable at each level of the second predictor and vice versa. Statistical significance was accepted at the Bonferroni adjusted alpha level.

Results

Evaluation of AFB1 dissipation kinetics under the different incubation conditions. The significant decrease in extractable AFB1 concentrations indicates that AFB1 degradation occurred in all investigated soils and incubation conditions (Fig. 1, Table 2). There were significant differences in terms of c/c_0 at the end of the 28-day incubation (Fig. 1a,b) between incubation conditions ($F(2,12) = 72.2$, $p < 0.001$). Overall, the order of AFB1 dissipation rate in both soils decreased in the order: PD > MD > C (Fig. 1a,c). The SFO dissipation rate constant in the UV-irradiated soils was slightly faster than microbial degradation by about 3% for the sandy loam and 17% for the clay soil (Fig. 1a,c). Dissipation was significantly lower in the sterile controls than in soils subjected to microbial degradation by about − 65% (sandy loam) and − 39% (clay), and in soils subjected to photodegradation by about − 66% (sandy loam) and − 48% (clay). At the end of the 28-day incubation, the c/c_0 was significantly lower in the sandy loam soil than in the clay soil ($F(1,12) = 71.0$, $p < 0.001$, Fig. 1a,b). AFB1 dissipation rate was higher in the sandy loam soil than in the clay soil by about 89%, 67% and 9% for the MD, PD and C, respectively (Fig. 1a,c). Further, a significant interaction between soil and degradation condition ($F(2,12) = 5.8$, $p = 0.017$) was found indicating that the dissipation kinetics derived from the degradation conditions was dependent on the soil type or vice versa. In this context, post-hoc analyses (see SI-4 Statistical analyses) had shown that AFB1 dissipated significantly faster in the sandy loam soil than in the clay soil for the MD ($F(1,12) = 31.7$, $p < 0.001$) and PD setup ($F(1,12) = 46.2$, $p < 0.001$) while the differences between the two soils incubated under C conditions were only marginally significant ($F(1,12) = 4.7$, $p = 0.051$).

Effects of initial AFB1 concentration on microbial degradation. AFB1 dissipated to varying degrees in the two tested nonsterile incubated soils at the different AFB1 fortification levels (Fig. 2, Table 2). The dissipation speed in terms of c/c_0 at the end of 28-days incubation (Fig. 2a,b) was significantly different between the soils ($t(26) = -12.0$, $p < 0.001$) and AFB1 fortification levels ($t(26) = -2.2$, $p = 0.040$). Further,

Type	Soil	AFB1 concentration level ($\mu g\,kg^{-1}$)	k_{SFO} (d^{-1})	R^2	DT_{50} (d)
MD	R01A	0.5	0.034	0.867	20
		5	0.036	0.964	19
		50	0.034	0.977	20
		250	0.033	0.977	21
		500	0.03	0.901	23
	L6S	0.5	0.014	0.772	48
		5	0.015	0.833	48
		50	0.018	0.907	37
		250	0.02	0.933	35
		500	0.016	0.885	43
PD	R01A	50	0.035	0.867	20
	L6S	50	0.021	0.888	32
C	R01A	50	0.012	0.842	56
	L6S	50	0.011	0.861	65

Table 2. Parameters of AFB1 dissipation kinetics for microbial degradation (MD), photodegradation (PD) and the sterile control in darkness (C): AFB1 SFO dissipation rates (K_{SFO}) and 50% dissipation times (DT_{50}) and adjusted coefficient of determination (R^2).

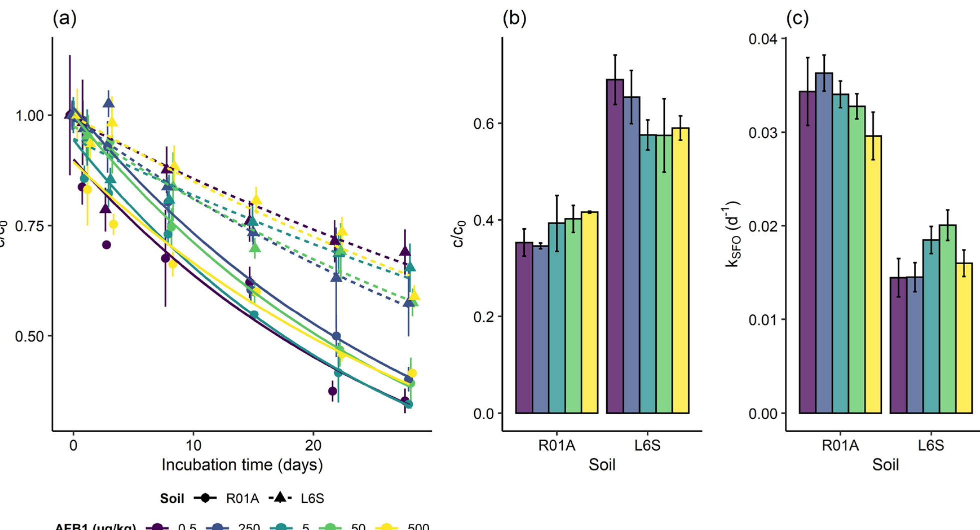

Figure 2. AFB1 dissipation for the sandy loam soil ("R01A") and clay soil ("L6S") amended with 0.5, 5, 50, 250 and 500 $\mu g\,kg^{-1}$ AFB1 and incubated under nonsterile (MD) conditions. Curves showing single first order kinetic model fitted to data (**a**), normalized AFB1 concentration c/c_0 at the end of 28-days incubation (**b**) and single first order dissipation rate constants (**c**).

the significant interaction between soil type and AFB1 fortification level ($t(26) = 2.8$, $p = 0.009$) indicates that the concentration dependant AFB1 dissipation was differently affected by the two soil types. Post-hoc analyses (see SI-4 Statistical analyses) showed that, there was a significant positive relationship between the AFB1 fortification level and the c/c_0 ratio for the clay soil ($F(1,26) = 4.7$, $p = 0.04$), while a marginally significant negative relationship was observed for the sandy soil ($F(1,26) = 3.5$, $p = 0.074$). The negative relationship between AFB1 fortification level and dissipation rate constant was consistent for the whole fortification range in the sandy loam soil (Fig. 2a,c). In contrast, for the clay soils the dissipation rate increased with increasing AFB1 fortification levels from 0.5 to 250 $\mu g\,kg^{-1}$ and then decreased at the highest level (500 $\mu g\,kg^{-1}$) almost to the level of the dissipation rate of the first two levels (0.5–5 $\mu g\,kg^{-1}$).

AFB1 dissipation processes and formation of AFB2a. A constant decrease of the extractable AFB1 fraction and a constant increase of the non-quantifiable fraction was observed for both soils and all incubation

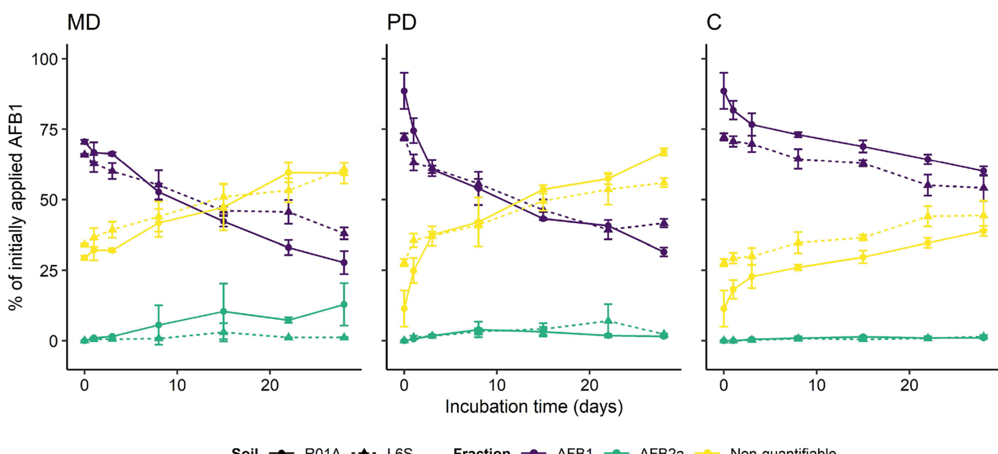

Figure 3. Processes of AFB1 dissipation for the sandy loam soil ("R01A") and clay soil ("L6S") incubated (28 days) under dark-abiotic (C), UV irradiated (PD) and nonsterile (MD) conditions. Extractable AFB1 (purple), extractable AFB2a (cyan) and non-quantifiable (yellow) fractions are given as percentage of initially applied AFB1. Results are expressed as mean ± standard deviation (n = 3).

conditions during the 28-days incubation (Fig. 3). AFB2a was detected as a transformation product under all degradation conditions, while no AFG2, AFG1 or AFB2 was found (see SI-3 Chromatographic data, Fig. 3). The transformation rate of AFB2a differed between the two soils and the three different degradation conditions. At the end of incubation period 12.9 ± 7.6 (MD), 1.5 ± 0.4 (PD) and 1.0 ± 0.3 (C) % of the initially applied AFB1 was found as AFB2a fraction in the sandy loam soil and 1.1 ± 0.2 (MD), 2.3 ± 0.2 (PD) and 1.4 ± 0.6 (C) in the clay soil. Considerably more AFB2a was found in the MD than in the PD and C samples but the variation was extremely high i.e. 50% of the samples had a coefficient of variation greater than or equal to 33% and 25% of the samples had a coefficient of variation greater than or equal to 52%. In particular, the MD samples showed a very high coefficient of variation up to 128% (R01A, MD, day 8). Only trace concentrations of AFB2a were detected in the sterile control. Throughout the 28 days incubation period, a higher AFB2a formation rate was observed for the MD setup in the sandy loam soil than in the clay soil (Fig. 3). This pattern was not observed for the C and PD conditions, where AFB2a concentrations were nearly equal for both soils, with one exception in the clay soil in PD on day 22, where the AFB2a concentration in the clay soil was 4 times that of the sandy loam soil. However, the relative standard deviation of this time point was 86%. A steady increase in the non-quantifiable fraction was observed over time for all soils and treatments. At the end of incubation 59.4 ± 3.7 (MD), 67.0 ± 1.3 (PD) and 38.9 ± 1.8 (C) of the initially applied AFB1 was found as nonextractable fraction in the sandy loam and 60.9 ± 2.2 (MD), 56.0 ± 1.6 (PD) and 44.4 ± 5.2 (C) in the clay soil. For the MD and PD, this fraction was the most significant at the end of the incubation experiment. The non-quantifiable fraction was nearly the same for both soil for the MD. However, for the PD the non-quantifiable fraction was considerably higher in the sandy loam soil than in the clay soil, while the opposite pattern was observed for the sterile control.

Discussion

Dissipation of AFB1 and formation of AFB2a occurred in all soils and under all incubation conditions, and the dissipation rate was significantly affected by soil type and degradation scenario. In both soils, the rate of AFB1 degradation in the PD- and MD-treated soils was of the same order of magnitude but was significantly higher than in the controls, as expected. However, the AFB1 dissipation kinetics observed for the PD and MD are much slower than in previous studies. In contrast, a considerable AFB1 dissipation was observed in the abiotic control. This is contrary to the general assumption that aflatoxins are almost recalcitrant to abiotic degradation in soil[6].

When subjected to microbial degradation, AFB1 dissipated with DT_{50} values of 19–23 days for the sandy loam soil and 35–48 days for the clay soil. These are much higher than the DT_{50} value of 4.1 obtained by Accinelli et al.[5], who used a similar fortification level of 10 $\mu g\,kg^{-1}$. Even at concentrations thousands of times higher (10−50 $mg\,kg^{-1}$), AFB1 could no longer be detected in less than 6 days[19,20]. One reason for the discrepancy between the dissipation rate in this study and other studies may be differences in the soil moisture conditions as this greatly affects the physiological state of microorganisms and the functionality of soil enzymes[53]. In former studies, a moisture content of 80-100% field capacity was reported[5,19,20], other to the 40% in this work. In this regard, a recent study[54] observed significantly lower microbial degradation of AFB1 in an artificial soil at 30% compared to 50% WHC. It should be noted that AFs degradation in real scenarios may take place under dryer environmental conditions. Therefore, the rapid degradation rates with DT_{50} of < 5 days reported previously[5,19,20] may underestimated the persistence of aflatoxins in the soil. Another reason for the discrepancy between the dissipation rate in this study and others is that reference soils from the European region were used in this study and hence it is unlikely that the microorganisms living in these soils have ever been exposed to AFs. Thus, the

enzymes involved in AFB1 degradation may be less effective than the enzymatic repertoire of microbes regularly exposed to aflatoxins.

The dissipation of AFB1 subjected to photolytic degradation was comparable in magnitude to microbial degradation. In the present study, DT_{50} values of 20 days for the sandy loam soil and 32 days for the clayey soil were observed for the photolytic degradation. These DT_{50} values are much higher than for photodegradation in other food or liquid matrices which are in the range of few minutes to hours[55]. This discrepancy is attributable to the high light attenuation effect of soil, as a soil layer as thin as 0.5 mm is already sufficient to block about 95% of the incident light[56]. Thus, it is expected that photolytic degradation is mainly limited to AFB1 contaminated material lying on top of the soil and the top layer of the soil. AFs are expected to accumulate mainly in the soil surface layer[26], thus photodegradation is likely to be of great importance for the degradation of AFs in contaminated soil.

In the sterile controls, a significant dissipation of AFB1 was observed with DT_{50} of 56 days for the sandy loam soil and 65 days for the clay soil. Furthermore, the presence of AFB2a in the sterile controls suggests that the dissipation of AFB1 observed is at least partly due to chemical degradation. This is contrary to the general assumption that AFs are almost recalcitrant to abiotic degradation in soil[5,6]. However, it is already known that the conversion of AFB1 to AFB2a can occur nonenzymatically in the presence of organic acids[36,57,58] that are also present in soil matrices[59]. Thus, chemically mediated degradation may be one of the underlying mechanism for the formation of AFB2a in the abiotic controls. In addition, it is possible that the soil enzymes were not deactivated during autoclaving[60], so that degradation of AFB1 may also have occurred by intact soil enzymes. Contaminated plant material is frequently incorporated into the soil post-harvest in the dry season[4,28] with limited microbial activity. Soil enzymes often remain active during drought[61] thus biochemical degradation could play an important role in the decomposition of AFs in the soil.

Regardless of incubation conditions, the degradation rate of AFB1 was significantly slower in the clay soil as compared to the sandy loam soil. These soils differ in physicochemical and microbial properties such as texture, organic carbon content, pH and microbial biomass and activity (Table 1). Although the microbial biomass (C_{mic}) and activity (BR, SIR) was around 2–3 times higher in the clay soil as compared to the sandy loam, the microbial dissipation of AFB1 was significantly lower in the clay soil, by about 89% compared to the sandy loam soil. This suggests that soil texture affected the availability of AFB1 for microbial degradation which is consistent with the results of Angle[20]. Medium strong sorption of AFB1 to soil organic carbon has been reported[21,22]. However, in this study, both soils are below 2% organic carbon content, and thus not considered as organic soils in which a higher probability of interaction between aflatoxin B1 and organic carbon would be expected. In addition, soil enzymes can also be sorbed to clay minerals in the soil[62], restricting their activity. AFB1 is relatively stable in the pH range of the soils studied (5.4 and 7.3) [63]. However, it was found that the binding strength of AFs[64] and soil enzymes[65] to clay minerals decreases significantly with increasing acidity[64,65]. To scrutinize the actual influence of soil pH on the bioavailability to soil microbes and thus on AFB1 biodegradation rate, further studies are needed on other soils at different pH gradients. Soil is known to attenuate light transmission, however the degree of this effect is driven by the soil texture, namely organic carbon and clay minerals. Organic substances such as humic substances and organic ions can act as photoquenchers that delay the photodegradation of a substance[39]. The substance to be degraded and the photoquenching organic ion can be sorbed together on the surfaces of the clay minerals, thus keeping the organic cations and the organic matter at an optimal distance and orientation for the energy transfer processes[66]. The clay mineral itself can also provide photostabilization by charge transfer from the excited organic molecules to Fe^{3+} ions in the crystal structure of the clay mineral[66–68]. However, it remains to be clarified which processes were actually responsible for the reduction in the dissipation rate of photolytic degradation.

It was found that the initial concentration of AFB1 affected the microbial degradation. A significant increase in degradation rate with increasing AFB1 concentration was observed for the clay soil (with a sharp decrease at the highest concentration), while for the sandy loam soil AFB1 concentrations had a marginally significant negative effect on degradation. In this context, Angle[20] observed a slightly reduced mineralization rate during the first 20 days in a silt loam soil amended with 10 $mg\,kg^{-1}$ compared to an amendment of 50 $\mu g\,kg^{-1}$. The same group also observed a negative effect of AFB1 (1, 100, 10,000 $\mu g\,kg^{-1}$) on the population of bacteria, actinomycetes and fungi in an agar medium and in a silt loam soil during the first 28 days after AFB1 application[8]. While these negative effects could be confirmed for the sandy loam soil, the opposite is was observed for the clay soil. This discrepancy may be explained by the interrelationship between sorption/desorption of AFB1 to clay minerals[19,20,23–26] and humic substances[21,22] and the effect on the bioavailability. As the desorption/adsorption coefficient of a given substance is a function of the substance concentration, there is consequently a higher fraction of AFB1 dissolved in soil pore water and a lower fraction adsorbed to sorption sites. Thus, the increase in dissipation rate over the first four AFB1 fortification levels (0.5–250 $\mu g\,kg^{-1}$) could be due to the increase in bioavailable concentration. At the highest level, the bioavailable concentration may surpassed the lowest concentration with detrimental effects on the microbial community, resulting in a decline of the dissipation rate. This proposed mechanism cannot be conclusively demonstrated from the present results. A classical ecotoxicity assay for the dose-dependent effects of AFB1 on microbial activity, biomass, and community structure could provide information on the dose-dependent effects on the rate of degradation.

Mass balance analysis showed that a large portion of the dissipated AFB1 was contained in the non-quantifiable fraction for all incubation conditions. However, it is unclear to what extent this non-quantifiable residue is due to volatilization of the parent compound, complete mineralization to CO_2, formation of bound residues, or incorporation of AFB1 carbon into microbial biomass. Volatilization as a cause for the increase in the non-quantifiable fraction seems not plausible, since no aflatoxin is known to be volatile under normal conditions (20 °C, 1 atm). In previous studies, only minor mineralization of AFB1 was observed in nonsterile soils, namely 14% in 112 days[19] and 1.4 to 8.1% in 112 days[20], while DT_{50} values $<$ 5 days were observed. Significant mineralization therefore remains unlikely compared to the other reasons given previously. Incorporation into the microbial

biomass seems unlikely as an exclusive process in light of the fact that a significant non-quantifiable fraction was also detected in the sterile soils (C and PD). Therefore it is likely that the discrepancy between the DT_{50} determined in the present study and in previous studies was due to a formation of bound residues that could not be removed from the soil matrix by the extraction procedure. The bound residues may not only include the parent compound AFB1, but also the metabolites formed. Hence, it is also possible that the metabolites formed could not be extracted by the extraction procedure used. A classic radiotracer analysis using radiolabelled standards or the application of further extraction steps or more sophisticated analytical methods, which are also able to detect large parts of the non-extractable residues[69], could provide further information on the fate of AFs in the soil.

Conclusion

The present study focussed on the degradation and transformation processes contributing to the dissipation of AFB1 in soil, namely microbial degradation and UV light-induced photodegradation. AFB1 dissipated in all soils and incubation conditions and AFB2a was detected as metabolite. The results clearly indicated that the dissipation of AFB1 was significantly affected by the incubation conditions, soil type and initial AFB1 fortification level. The largest fraction of dissipated AFB1 was found in the non-quantifiable fraction indicating that soil-bound residues of the parent compound and/or metabolites were formed. Regardless of the soil tested, a clear pattern emerged in which AFB1 dissipation and AFB2a formation were significantly higher in PD and MD treated soils than in the sterile control. AFB1 dissipation rates for the PD and MD treatments were of a similar magnitude, with the PD treatment being slightly faster. Due to the low penetration depth of UV light in soil, photodegradation is expected to be limited to the uppermost soil layers, so that AFB1 degradation in deeper soil layers is likely to be dominated by microbial degradation. A negative effect of initial concentration on AFB1 dissipation rate was observed for the sandy loam soil but not for the clay soil, which is probably explained by the sorption-induced reduction in bioavailability due to the higher clay mineral content. Although the dissipation rates in the sterile controls were much lower than microbial and photodegradation, biochemical degradation in dark could play an essential role in the degradation of AFB1 when conditions are unfavorable for microbial degradation, such as during extreme drought. Altogether, these results suggest that photolytic and microbial degradation processes are particularly important in the breakdown and deactivation of AFB1 in soil, although these processes depend on the soil properties. The results of this study contribute to a better understanding of the fate and importance of AFs as micropollutants in the environment and illustrate the importance of soil properties for the dissipation processes of AFB1.

Data availibility

All data generated or analysed during this study are included in this paper and its supplementary information. Additional data related to this paper may be requested from the authors on reasonable request.

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Author contributions

J.A. designed the study, conceived the experiments, conducted data analysis, wrote and revised the manuscript; K.M. was involved in the study design, acquired funding and revised the manuscript.

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Chapter 5

Consequences of Aflatoxins for Soil Health

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Article

Soil Microbial Responses to Aflatoxin Exposure: Consequences for Biomass, Activity and Catabolic Functionality

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Abstract: Aflatoxins (AFs) are fungal secondary metabolites frequently detected in soil that exhibit in vitro toxicity to certain soil microorganisms. However, microbial responses at different levels and in complex systems such as the soil environment have not been systematically studied. Therefore, we investigated multiple microbial responses in two different soils (sandy loam and clay) to aflatoxin B1 (AFB1) at environmentally relevant concentrations (0.5–500 μg kg^{-1}) during a 28-day incubation. General microbial parameters for biomass (microbial biomass carbon and ergosterol), activity (glucose-induced and basal respiration), and catabolic functionality (substrate utilization patterns) were assessed. We observed minor and transient effects in both soils. In sandy loam, we found negative effects on activity and catabolic functionality with increased metabolic quotient, while clay soil exhibited stimulation for the same parameters, suggesting a hormetic effect due to reduced bioavailability through sorption onto clay minerals. Our results indicate that AFB1 does not pose a threat to general microbial indicators under the test conditions in soils without previous AF contamination. Given the toxic potential of AFs to specific microorganisms, further studies should investigate responses at higher taxonomic and functional levels in natural environments of aflatoxigenic fungi, such as tropical soils, and including additional physicochemical stressors.

Keywords: aflatoxin; effects; soil microbial activity; soil microbial biomass; catabolic functionality

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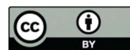

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1. Introduction

Aflatoxins (AFs) are toxic secondary metabolites synthesized by certain fungal strains of the anamorph genus *Aspergillus*. Aflatoxigenic fungi naturally occur in a wide variety of environmental matrices, including soil and plant residues [1–4] and AF concentrations ranging from 10^{-2} to 10^{2} μg kg^{-1} have been reported [1]. The major part of the life cycle of *Aspergilli* fungi takes place in the soil as they do not only colonize living plant tissue, but also grow saprophytically on plant debris [5]. These habitats serve as a reservoir for the fungus, allowing it to overwinter, and under favorable conditions resume growth with the potential to infest plants and crops [2,5]. In soil and decaying vegetation, these toxigenic fungi can produce AFs, thus introducing AFs into the soil [1,6]. In addition, agricultural activities such as the incorporation of contaminated crop residues and manure [7–10] may result in inputs of aflatoxigenic fungi and AFs into the soil system beyond the natural levels. It has been reported that the soil microbiome and its associated functions can be impacted by the presence of natural toxins, including plant secondary metabolites such as phenolic compounds [11–13]. Thus, the introduction of AFs to the soil has the potential to alter the ecological balance and pose a risk to the integrity of the soil microbiome and thus to soil health [14].

In the establishment and growth of mycotoxin-producing fungi in the soil, they compete with other living organisms for the same resources [6]. The production of AFs could be a response to this microbial competition and thus part of the ecological strategy of aflatoxigenic fungi. This is supported by the fact that sclerotia and condidia spores, the structures that have to survive in the soil for a long time, have a particularly high concentration of

AFs [15]. Furthermore, increased in vitro AFs production was observed in the presence of competing soil microbes, including Gram-positive bacteria, Gram-negative bacteria, yeasts and filamentous fungi [16–18]. Other studies have shown that the production of AFs was unaffected, decreased, or even completely inhibited in the presence of filamentous fungi and Gram-positive bacteria [16,18]. Agricultural practice can significantly increase the AF level in soils beyond natural levels, with the potential to affect the soil microbiome and the soil functions it provides, thus altering the ecological balance of the soil [6,14].

The microbial response to a chemical exposure can be investigated at multiple scales. First, the response can be tested in vitro by exposing the test organism directly to the chemical stressor and excluding influencing factors such as the the natural environment. In this regard, growth inhibition was observed for some Gram-positive bacteria including *Bacillus*, *Nocardia*, *Clostridium*, and *Streptomyces* in agar media supplemented with AFs (30 and 100 mg L^{-1}), while other common Gram-positive and Gram-negative bacteria, fungi, algae, and protozoa were unaffected [19,20]. However, the tested concentrations are well above observed levels in contaminated agricultural commodities [1]. At concentrations closer to environmental levels, Angle and Wagner [21] observed inhibitory effects on native soil microorganism in two experiments: First, they observed a continuous decrease of 34–38% in the propagules of viable fungal, bacterial, and actinomyces populations isolated from a uncontaminated silt loam soil and cultured in agar medium spiked with 1, 100, 1000, and 10,000 µg AFB1 L^{-1} compared to the control. Next, they observed negative effects for the viable fungal, bacterial, and actinomyces populations isolated from a AFB1 contaminated silt loam soil (1, 100, 1000, 10,000 µg AFB1 kg^{-1}) that were cultured in agar medium. These effects occurred after two weeks of exposure and persisted for nearly six weeks. However, both the concentrations and conditions for the microbes tested using in vitro laboratory tests may not be representative of the conditions they encounter in their natural habitat, i.e., the soil environment [22].

Although in vitro studies provide key evidence on specific responses, they may not be representative of complex environmental systems since other influencing external factors are excluded [22]. In addition, less than 1% of the total microbiome can be cultured on agar media [23]. For the soil microbiome, the study of such responses should include the soil as a whole and evaluate responses at multiple levels [24]. How the microbial biomass and its composition change over the course of AF exposure in the soil has not yet been systematically investigated. The microbial response may manifest itself in the altered physiology of the microbiome, e.g., respiratory activity and substrate utilization efficiency. In this context, Angle and Wagner [21] found a significant reduction in the basal respiration rate (i.e., microbial CO_2 production without substrate addition) of the soil microbiome at the highest AFB1 level of 10,000 µg AFB1 kg^{-1}, as compared to the control. At lower levels, respiration was not significantly different from the control. Basal respiration is mainly determined by substrate availability in soil, but also depends on physiological status and microbial maintenance requirements. Therefore, basal respiration can be considered as an indicator of integrated metabolic activity, but not of active microbial biomass, as it only captures the respiration of currently active microbes [25]. The application of a readily available substrate (such as glucose) prior to respiration measurement (substrate-induced respiration) stimulates a large fraction of the inactive microbiome, so that the respiratory response of the original soil microbial biomass can be investigated [26]. The microbial response to AF exposure may be reflected in a change in the catabolic functionality of the microbiome, which can be evaluated by carbon source utilization patterns [27,28]. Thereby, the quantity of utilized carbon sources reflects the abundance of microbial biomass that is able to utilize the corresponding carbon source [29]. It is assumed that the range of carbon sources utilized reflects the functional diversity of the microbial community [30]. The application of antibiotics to selectively inhibit fungi, prior to substrate-induced respiration, allows the investigation of the response of the fungal fraction of the microbiome [27,31,32].

The aim of the present study was to systematically investigate the soil microbial responses due to AF exposure at different levels, including the biomass, activity and

catabolic functionality. Furthermore, the extent to which these effects are influenced by physicochemical soil parameters was also investigated. For this purpose, sandy loam and clay soil were contaminated with AFB1 ranging from 0.5 to 500 µg kg^{-1} and then incubated for 28 days. At discrete time points, different soil microbial parameters were assessed: total microbial biomass (via the chloroform-fumigation–extraction method), total fungal biomass (via the biomarker ergosterol), and substrate utilization patterns of the total microbial (MicroResp) and fungal communities (FungiResp). Microbial and ecophysiological ratios were calculated to detect changes in the composition or physiological state of the microbial community [24,25]. Due to the known toxicity of AFB1 on soil microbes [19–21], we expect (i) a dose-driven reduction in the microbial biomass carbon (C_{mic}) and fungal biomass marker ergosterol (ERG), and overall reduction in multiple-substrate-induced respiration for the whole microbial and fungal communities. Since AFB1 is more toxic to soil bacteria than fungi [19,20], we expect (ii) changes in the activity and biomass composition of the microbiome towards an increase in fungal fraction. Furthermore, we hypothesize stress-induced (iii) changes in the physiological state towards an increased basal-to-substrate induced respiration ratio, increased metabolic quotients, and the reduced utilization of more complex carbon substrates. Since clay minerals strongly bind AFs [33–38], we assume that (iv) the toxic effects of AFB1 are less pronounced in the more clayey soil, as a result of reduced bioavailability.

2. Materials and Methods

2.1. Chemicals and Reagents

The AFB1 stock solution used for sample fortification was prepared by dissolving crystalline AFB1 (from *Aspergillus flavus*, Sigma-Aldrich, St. Louis, MO, USA) in acetonitrile according to the procedure decribed by Albert and Muñoz [39]. Ultrapure water was used throughout all work (produced by a Milli-Q-water purification system, 18.2 MΩ cm^{-1}, EASYpure II, Millipore Bedford, MA, USA). Methanol (MeOH) used for ERG extraction and chromatography was of HPLC grade (Carl Roth, Karlsruhe, Germany). ERG used for external calibration was of LC grade (purity $\geq$ 95.0%, Sigma-Aldrich, Taufkirchen, Germany). The carbon substrates used for respiration experiments were D-glucose (purity $\geq$ 99.5%), D-galactose (purity $\geq$ 98%), L-alanine (purity $\geq$ 98.5%), N-acetyl-D-glucosamine (purity $\geq$ 98%), α-cyclodextrin (purity $\geq$ 98%), and trisodium citrate (purity $\geq$ 99.5%), purchased from Carl Roth (Karlsruhe, Germany). The substrate γ-aminobutyric acid (purity $\geq$ 99%) was obtained from Sigma-Aldrich (Taufkirchen, Germany). The bacterial inhibitor bronopol (purity $\geq$ 98%) was purchased from Thermo Scientific (Schwerte, Germany).

2.2. Description of Test Soils

The experiments were carried out using the reference soils "RefeSol 01-A" (Fraunhofer IME, Schmallenberg, Germany) and "LUFA 6S" (LUFA, Speyer, Germany). Refesol 01-A is a strongly acidic, very light humic sandy loam soil, and LUFA 6S is a light humic and slightly alkaline clay soil (Table 1). Samples were collected from the upper layer, i.e., at 0–20 cm (LUFA 6S) and 0–25 cm (RefeSol 01-A), of organically managed arable soils from suppliers, and conditioned according to the requirements of OECD Guide 217 [40]. These soils were selected to cover a range of physicochemical properties thought to affect the bioavailability of AFB1 to soil microbes, i.e., organic carbon content, pH, and soil texture. Soil samples were prepared (removal of vegetation, larger soil organisms, and stones, and sieving through a 2 mm sieve) within one week of sampling and stored at 4 °C under aerobic and dark conditions for less than one month until use in the incubation experiments. Before conducting the main experiments, the moisture of both soils was adjusted to 40% of the maximum water holding capacity to ensure optimal microbial conditions [40]. The moisture-adjusted soils were incubated in the dark at 20 °C under aerobic conditions for 1 week to establish the equilibrium of microbial metabolism after the change from storage to incubation conditions. The total microbial biomass carbon (C_{mic}) of the soil

microbiome prior to conducting the incubation experiment was determined using the chloroform fumigation extraction method (see Section 2.4).

Table 1. Physicochemical and microbial properties of the tested soils.

Property	RefeSol 01-A	LUFA 6S
Soil type	sandy loam	clay
Sand (%)	70.5	23.3
Silt (%)	26.1	35.5
Clay (%)	3.4	41.2
C_{org} (%)	0.9	1.7
N_{tot} (%)	0.08	0.18
WHC (%)	29.3	42.4
pH (0.01 M $CaCl_2$)	5.4	7.3
C_{mic} (mg kg^{-1})	95 ± 15	267 ± 8

2.3. Aflatoxin B1 Concentrations, Soil Incubation and Sampling

Incubation experiments were carried out at four AFB1 levels with 5, 50, 250 and 500 µg kg^{-1} and a blank free of AFB1. These concentrations were chosen in line with previously reported concentrations found in soil and decaying plant material [1]. Soils were prepared according to the procedure described by Albert and Muñoz [39]. Briefly, aliquots of 3 kg soil (dry weight) were spiked by extensive shaking with AFB1-coated quartz sand (0.1–0.315 mm, acid washed) as a solid carrier at a mass ratio of 1% [40] in a securely sealed polypropylene bag. Quartz sand was coated with AFB1 using a spiking standard solution of a concentration of 500 mg L^{-1} AFB1 in acetonitrile. Methanol was avoided as a spiking solvent to prevent the formation of artifactual methoxy species of AFB1 [41]. The solvent was allowed to evaporate for 1h before the spiked sand was mixed with soil to avoid the potential effects of the solvent carrier on the soil microbiome [42]. The blank was prepared using the same procedure but with pure acetonitrile. Soils were then split into aliquots for the incubation experiments. For the determination of ERG and C_{mic}, 100 g (dry weight) aliquots were incubated in 200 mL polypropylene screw-cap beakers equipped with a polyester filter floss in the cap in order to maintain aerobic conditions while minimizing the evaporation of water. To assess the microbial and fungal respiration and substrate utilization patterns as an indicator of the catabolic profile of the microbial and fungal communities, spiked or control soils were filled into 96-deep-well plates. Each plate contained a single soil at a single contamination level for a discrete sampling date. Half of the plate was then used for the analysis of the microbial catabolic profile (MicroResp) and the other half for the fungal catabolic profile (FungiResp). The filled plates were covered with Parafilm to minimize water loss while ensuring aerobic conditions. Filled incubation beakers and plates were incubated at 20 °C in the dark, and single samples were removed and analyzed at 0, 1, 3, 8, 15, 22, and 28 days of incubation. ERG and C_{mic} contents were determined in triplicate. The respiration and catabolic profiles were assessed in duplicate. The study design and experimental workflow are shown in Figure 1.

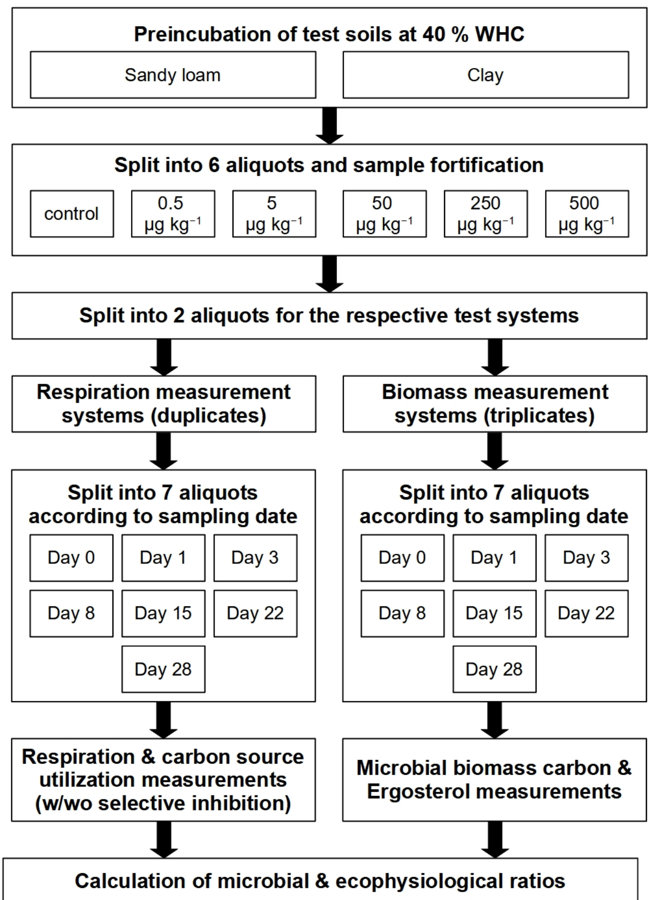

Figure 1. Flowchart describing the study design and experimental set up.

2.4. Soil Microbial and Fungal Biomass

Soil microbial biomass carbon (C_{mic}) was determined by the chloroform-fumigation method [43]. Briefly, fumigated (24 h, chloroform) and nonfumigated soils (20 g, fresh weight) were extracted with 80 mL of 0.5 M K_2SO_4 by orbital shaking for 30 min. Extracts were filtered through a paper filter (MN 619 eh1/4, Ø: 110 mm, MACHEREY-NAGEL, Düren, Germany) and stored at −20 °C until further analysis. The filtered soil extracts were analyzed for dissolved organic carbon content with a TOC analyzer (multiNC 2011S, Analytik Jena AG, Jena, Germany). Microbial biomass carbon was calculated as the difference in carbon content between fumigated and nonfumigated values, employing a conversion factor of 0.45 [44].

The fungal cell membrane component ergosterol as an indicator of fungal biomass was extracted from soil by physical disruption according to the method described by Gong et al. [45], using a modified HPLC-UV method. Briefly, 4 g (fresh weight) of soil was extracted with 6 mL of methanol by orbital shaking (1 h, 320 rpm) in the presence of 4 g of acid-washed glass beads (2 g 212–300 µm and 2 g 710–1180 µm) followed by centrifugation at 2190× g for 10 min. The extracts were filtered through syringe filters (0.2 µm, PET) and stored at −20 °C until measurement. HPLC analysis was performed on an Agilent 1200 series (Agilent, Santa Clara, CA, USA) system (G1311A Quaternary pump, G1322A degasser, G1329A autosampler) equipped with a column oven (Jetstream 2

column thermostat, KNAUER, Berlin, Germany) and UV detector (G1314B, Agilent, Santa Clara, CA, USA). Chromatographic separation was achieved on a LiChrospher 100 RP18 5 µm 4.6 × 250 mm column (CS Chromatographie-Service, Langerwehe, Germany) at 38 °C using isocratic elution mode, consisting of a mixture of methanol/acetonitrile (95:5, v+v) at a flow rate of 1.7 mL min^{-1}. The injection volume was set to 100 µL. ERG was detected at an absorbance wavelength of 282 nm and quantified by external standard calibration in the range of 0.05–5 mg L^{-1} (adj. R^2 = 0.999, Appendix A, Table A1). The instrumental limit of detection (LOD) and limit of quantification (LOQ), calculated according to the calibration method (DIN 32645, 2008), were 0.03 ± 0.01 and 0.09 ± 0.03 mg L^{-1}, respectively.

2.5. Determination of Microbial and Fungal Respiration and Catabolic Profiles

Analysis of the substrate utilization patterns as a proxy for the catabolic profiles of soil microbial and fungal communities was performed using the miniaturized soil respiration system MicroResp, as described by Campbell et al. [28]. This method was further developed by Sassi et al. [27] into the so-called FungiResp method by using the selective bacterial inhibitor (Bronopol) to obtain the catabolic profile of the fungal fraction of the microbiome. The MicroResp method measures the microbial respiration rates induced by different carbon sources in a microplate-based respiration system [29]. Briefly, moist soil samples (adjusted to 30–60% WHC) with or without (basal respiration) carbon substrates were incubated in a 96-deep-well microplate for 6 h. CO_2 production was then evaluated by a pH-change-driven color reaction in an attached 96-well detection plate with agar gel containing the indicator dye cresol red [28]. This color change is proportional to the CO_2 evolved and is quantitatively measured by absorbance in a microplate reader at 572 nm. The following carbon sources were tested: the simple carbohydrates D-glucose and D-galactose; the amino acids L-alanine and γ-aminobutyric acid and the amino compound N-acetylglucosamine; the organic acid citric acid (as sodium citrate); and the complex carbohydrate α-cyclodextrin. These substrates were used due to their ecological relevance, their known occurrence in the soil environment (e.g., plant root exudates), and their ability to provide a sufficient range of structural complexity [46–48]. The respiratory response to the respective substrate addition reflects the proportion of active microbial biomass capable of utilizing the corresponding carbon source. Water was added to assess basal respiration. The substrates were prepared in ultrapure water at a concentration of 30 mg (g of soil water^{-1}) [28]. The less soluble substrates (L-alanine, N-acetylglucosamine, α-cyclodextrin) were prepared as stock solutions to deliver 7.5 mg (g of soil water)$^{-1}$ [28]. In order to assess the respiration of the soil fungi, 25 µL of the bacterial inhibitor bronopol (dissolved in ultrapure water) was applied to the 96-deep-well microplates to achieve a nominal spiking level of 78 µg g^{-1} (dry weight) [27]. Bronopol-spiked soils were preincubated for 1 h in order to induce sufficient inhibition prior to substrate application [27,32]. The catabolic profile of the whole microbial community was assessed by applying water instead of bronopol. After 1 h preincubation, the carbon substrates were distributed via 25 µL aliquots in a randomized block design to compensate for any edge effects on the 96-deep-well microplate [29]. Each deep-well microplate was sealed on a 96-well detection plate via a silicone seal (MicroResp, The James Hutton Institute, Dundee, UK) and incubated at 20 °C in the dark for 6 h. The absorbance of the detection plates was measured at 572 nm on an Infinite M200 plate reader (Tecan Trading AG, Männedorf, Switzerland) immediately before sealing (A_{t0}) and after 6 h incubation (A_{t6}). According the to the manufacturer's instruction, the absorbance values were normalized by dividing the A_{t6} readings by the A_{t0} readings and multiplying them by the average A_{t0} readings obtained across all wells within each plate (A_i).

$$A_i = \frac{A_{t6}}{A_{t0}} \times \overline{A_{t0}} \quad (1)$$

Normalized absorbance values were converted to the CO_2-C air fraction by the construction of a nonlinear calibration curve. A calibration curve was constructed from

normalized absorbance values versus the headspace C-CO_2 air fraction obtained from the 6 h incubation of 8-well strips from a breakable microplate (12 strips of 8 wells) using gas mixtures with a known CO_2-C air fraction (0.05–5%) and fitted to the inverse model provided by the manufacturer (adj. R^2 = 0.993, Appendix A, Table A2). The respiration rate (µg CO_2-C g^{-1} h^{-1}) was calculated by converting the 6 h CO_2-C air fractions to µg g^{-1} h^{-1} CO_2-C using gas constants and constants for headspace volume in the well (945 µL), fresh weight of soil per well (g), incubation time (h), and soil sample percent dry weight according to the manufacturer's protocol.

2.6. Soil Microbial Indices and Ecophysiological Ratios

When assessing the impact of a chemical on the microbiome, the characterization of the community structure and physiological state of the microbial community is crucial for a more comprehensive assessment of the environmental impact of a chemical stressor [24]. The ratio of ergosterol to microbial biomass carbon ($ERG{:}C_{mic}$) functions as an indicator for the fungal fraction of the total microbial biomass. Larger $ERG{:}C_{mic}$ ratios indicate an increase in the fungal fraction within the soil microbial community.

$$ERG : C_{mic} = \frac{Ergosterol}{C_{mic}} \tag{2}$$

Similarly, respiration ratios for the basal respiration ($BR_{fun}{:}BR_{mic}$) and the glucose-induced respiration ($GIR_{fun}{:}GIR_{mic}$) can be calculated as an indicator fungal fraction of the total microbial activity [25]. The basal-to-substrate ratio induced respiration function ($Q_{R,mic}$, $Q_{R,fun}$) acts as an indicator of the physiological state of the soil microbial community [25]. If the respiration rates inhibited by bronopol are used for calculation, the corresponding equivalent for the fungal fraction of the whole microbiome ($Q_{R,fun}$) is obtained.

$$Q_{R,mic} = \frac{BR_{mic}}{GIR_{mic}} \tag{3}$$

$$Q_{R,fun} = \frac{BR_{fun}}{GIR_{fun}} \tag{4}$$

The ratio between basal and SIR respiration is restricted to the range between 0 and 1 and indicates the respiration ratio between growing and potentially active microorganisms. Values close to one correspond to the absence of an increase in respiratory response due to substrate addition and thus the absence of potentially active microorganisms, indicating strong suppression due to environmental stress or disturbance [25].

The metabolic quotient ($qCO_{2,mic}$) is calculated from the basal respiration and microbial biomass and reflects the the energetic efficiency of a microbial community. The higher the ($qCO_{2,mic}$) value, the less efficient the microbial turnover as a result of a decrease in biomass and a simultaneous increase in CO_2. An increase in $qCO_{2,mic}$ is considered as an indication of stress [49–51]. Similarly, an equivalent of the metabolic quotient for the fungal fraction of the soil microbiome ($qCO_{2,fun}$) can be calculated from the basal respiration inhibited by bronopol (BR_{fun}) and the fungal biomass marker ergosterol (ERG).

$$qCO_{2,mic} = \frac{BR_{mic}}{C_{mic}} \tag{5}$$

$$qCO_{2,fun} = \frac{BR_{fun}}{Erg} \tag{6}$$

2.7. Data Analyses

Data processing, analyses, and visualization were conducted using R (version 4.0.3) with "tidyverse" [52] as the main package for data preparation and the "vegan" package [53] for multivariate statistics. Linear calibration curve fitting and the calculation of instrumental

LOD and LOQ were conducted with the “calibration” function implemented in the R package “envalysis” [54].

Concentration and time course effects of AFB1 on the soil microbial and ecophysiological parameters for the individual soils were investigated using multiple regression models with the continuous predictors “AFB1 concentration” and “Incubation time”. For the sandy loam soil, C_{mic} values near or below zero were found for day 0, so day 0 was excluded for the statistical analyses performed for C_{mic} and the ERG:C_{mic} ratio. To test whether the effect of AFB1 concentration on the respective response variables depended on incubation time, an interaction term (“AFB1 concentration: Incubation time”) was included. For all multiple regression models, assumptions were verified by diagnostic plots [55], i.e., the criterion of (i) normality was verified via residual quantile–quantile plots, (ii) homoscedasticity via scale location plots (square root of standardized residuals versus predicted values), (iii) absence of autocorrelation via autocorrelation plots, and (iv) absence of multicollinearity via the calculation of variance inflation factors (VIF). VIF values greater than 10 were considered problematic [56]. To compensate for experimental artefacts in the MicroResp setup (e.g., edge effects) [29,57], outliers were detected and removed by the median absolute deviation (MAD) method [58]. Response variables were transformed where appropriate to meet model assumptions using frequently applied and reasonable power transformations (to the power of −2, −1, −0.5, 0.5, 1, and 2). The optimal transformation parameter was determined by the Box–Cox transformation technique [59]. Thereby, the transformation parameter closest to the best lambda value and within the confidence interval was used to perform a transformation. The test statistics for each multiple regression model are available in the Appendix (Appendix D, Table A3).

Principal response curves (PRC) [60–63] were used to assess the temporal multivariate catabolic response for each AFB1 concentration level as deviations from the nonspiked control (Appendix B). Separate principal response curve analyses were performed for each bronopol-inhibited and noninhibited soil. Monte Carlo permutation tests were conducted to assess the significance of the effects of the explanatory variable (i.e., AFB1 concentration level) on the multivariate response using an F-type statistic based on the eigenvalue of the component [60,61]. The results of the Monte Carlo permutation tests are available in the Appendix (Appendix D, Table A4).

3. Results and Discussion

3.1. Biomass Responses to Aflatoxin Exposure

For the clay soil, a significant positive effect of AFB1 concentration ($p < 0.001$) and incubation time ($p = 0.017$) on C_{mic} was observed (Appendix D, Table A3, Appendix B, Figure A3). Moreover, the interaction between both concentration and time was significant ($p = 0.004$), indicating that the effect of AFB1 on C_{mic} was affected by the incubation time. The C_{mic} values increased by about 67% and 377% at the highest AFB1 level (500 µg kg $^{-1}$) from day 0 to 1 as compared to the control (Figure 2a). This effect was not further observed over the course of the incubation. For the sandy loam soil, C_{mic} content was significantly affected by incubation time ($p = 0.003$) with a tendency towards increased values at the end of incubation (day 28). C_{mic} was not significantly affected by AFB1 concentration ($p = 0.466$). However, at the end of incubation, C_{mic} values were reduced by about 29% (day 22) and 23% (day 28) at the highest concentration level (500 µg kg $^{-1}$) in comparison to the control.

The clay soil showed a significant negative effect of AFB1 concentration ($p = 0.009$) on ERG values, which was particularly pronounced until day 15 of incubation (Figure 2b, Appendix D, Table A3, Appendix B, Figure A3). However, at the end of incubation, ERG values were increased at the highest AFB1 concentration compared to the control. Furthermore, ERG values decreased slightly but significantly with incubation time ($p < 0.001$). In the sandy loam soil, no effect of AFB1 concentration on ERG values was observed ($p = 0.784$), except for day 22, where the ERG content in the control was lower than for the

highest AFB1 level (500 µg kg $^{-1}$). However, the ERG content significantly increased over time ($p < 0.001$), with levels at day 28 being approximately 60% higher than at day 0.

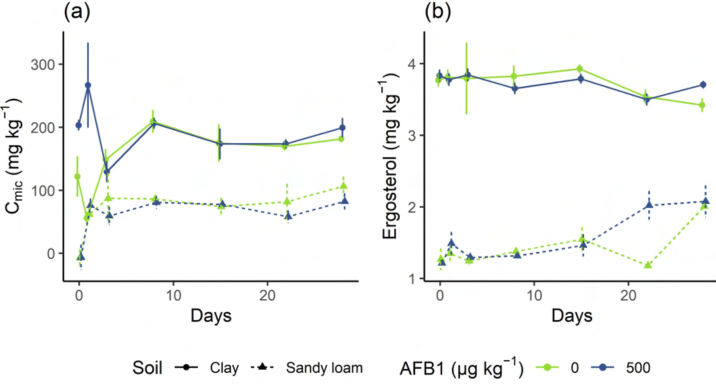

Figure 2. Microbial and fungal biomass responses to AFB1 exposure as a function of the incubation time. Curve plots showing the average values for microbial biomass carbon (**a**) and ergosterol as bioindicators for fungal biomass (**b**). The error bars represent the standard deviations.

It is unlikely that the AFB1-induced increase in microbial carbon biomass observed for the clay soil is due to the use of AFB1 as a carbon source to build microbial biomass, since the carbon provided by AFB1 application is multiple magnitudes lower than the increase in microbial biomass carbon. Even at the highest AFB1 dosage of 500 µg kg^{-1} and a 100% utilization rate of AFB1 carbon for microbial growth, the increase in microbial biomass carbon could be at most 327 µg kg^{-1}. This is much less than the observed increase in microbial biomass carbon of about 213 mg kg^{-1}. More likely, the presence of AFB1 may have affected the microbial physiological and biochemical properties and thus the fumability by chloroform and/or extractability of dissolved organic carbon released from the lysed cells. The applied AFB1 may have changed the K_2SO_4 extraction recovery by desorbing dissolved organic carbon released from microbial cells from the soil matrix, resulting in a change in the measured C_{mic} independent of the actual microbial biomass [64]. AFB1 is known to have a very strong sorption affinity to clay minerals by electron–donor–acceptor interactions between the two electron-rich carbonyl groups in the coumarin structure and electron-deficient or positively charged species located at the negatively charged surface of clay minerals [33]. Furthermore, AFs strongly interact with soil organic matter with log K_{OC} values ranging from 2.80 to 3.46 [65]. AFs with a double bond in the terminal tetrahydrofuran ring (AFB1, AFG1) have a higher sorption affinity than the saturated forms (AFB2, AFG2) [65]. This suggests that the terminal tetrahydrofuran ring is a major site of interaction with organic carbon compounds, while the coumarin ring is a site of interaction with clay mineral surfaces. In addition, nonpolar fractions of the molecule, i.e., the benzene ring and the conjugated system in the molecule, interact with aromatic fractions of the soil organic matter due to π–π interactions [66,67]. Thus, the DOC molecules present in nonfumigated samples may form DOC-AFB1-clay mineral structures, resulting in lower K_2SO_4 extraction efficiencies for DOC in the nonfumigated samples as a function of AFB1 concentration. In this context, the positive relationship between AFB1 concentration and dissolved organic carbon extracted from nonfumigated soils for days 0 and 1 (Appendix F, Figure A7) supports this mechanistic explanation. Furthermore, it was observed that the microbial biomass carbon calculated for day 0 in the sandy loam soil was near or below zero regardless of the AFB1 level (including the control). Because the near-zero C_{mic} observed in the control was not statistically different from the AFB1 contaminated soils, the absence of any measured microbial biomass could not be attributed to the toxic effects of AFB1. Rather, the near-zero concentrations on day 0 in the sandy loam are probably due to methodological

issues. In this regard, the extensive mixing of the soil during spiking may have resulted in cell lysis due to physical stress in the form of crushing by sand particles (sand content = 70.5%). However, such a decrease in C_{mic} at day 0 was not observed for the clay soil with a much lower sand content (23.2%). Since soil microbes strongly bind to soil clay minerals, they could be protected against these forms of physical stress in the clay soil.

Angle and Wagner [21] observed a decrease in the viable population of soil bacteria, fungi, and actinomyces, which is in contrast to the results observed in the present study. This discrepancy may be explained by differences in the methodologies. First, in the present study, we investigated the entire microbiome in the soil as a natural habitat. In contrast, Angle and Wagner [21] either inoculated extracted soil populations in AFB1-supplemented agar media or extracted the microbial population from AFB1-fortified soil matrix by phosphate buffer extraction followed by cultivation on agar media. It is known that the majority of soil microbes (>99%) are not cultivable using conventional agar cultivation techniques [23] and, thus, the successfully cultivated microbial consortium was not representative of the total phylogenetic diversity. Hence, the toxicity observed in the study of Angle and Wagner [21] affected only a few of the species that were surveyed. Second, when using an agar plate approach, the bioavailability of AFs is likely to be much higher than in soil matrices where soil components such as clay minerals and humic substances strongly interact with AFB1 [33,65]. These methodological differences could also explain why, in the same study, almost no negative effects of AFB1 were found on the respiration of the total soil microbiome, which is generally a more sensible parameter to assess the adverse effects of a substance as it may also show nonlethal effects on soil microbes.

3.2. Response of Microbial Activity to Aflatoxin Exposure

Irrespective of the soil tested, no significant effect ($p > 0.05$) of AFB1 concentration on the BR_{mic} and BR_{fun} was observed (Appendix D, Table A3, Appendix B, Figure A4). In addition, the BR_{mic} (Figure 3a) and BR_{fun} (Figure 3b) was not significantly affected by incubation time, except for BR_{fun} in the clay soil ($p = 0.008$). However, in the clay soil, BR_{mic} and BR_{fun} values were slightly decreased at the highest spiking level (500 µg kg^{-1}) compared to the control after the first week and especially at the end of incubation. Likewise, the GIR_{mic} and GIR_{fun} were not significantly affected by incubation time or AFB1 concentration in the sandy loam soil ($p > 0.05$, Appendix D, Table A3, Appendix B, Figure A4). In contrast, the GIR_{mic} was significantly positively affected by incubation time ($p < 0.001$) and AFB1 concentration in the clay soil ($p = 0.009$, Figure 3c). However, the interaction between time and AFB1 concentration was significant ($p = 0.009$), indicating that the effect of AFB1 on GIR_{mic} was affected by the incubation time, with a tendency for positive effects of AFB1 on GIR_{mic} at the beginning of the incubation period and slightly negative effects at the end of incubation. In this regard, GIR_{mic} and GIR_{fun} values were increased at the highest spiking level (500 µg kg^{-1}) compared with the control (Figure 3c,d). For the GIR_{fun} in clay soil, no effect of AFB1 concentration was found ($p = 0.08$, Figure 3d).

The results of the present study are in line with Angle and Wagner [21], who observed no effect of AFB1 application at similar AFB1 fortification levels (from 1 to 1000 µg kg^{-1}) on the basal respiration in a silt loam soil. This can be explained by the relatively high cation exchange capacity of the silt loam soil (14 meq $(100\ g)^{-1}$) [21], indicating a high content of clay minerals [68], a soil fraction that is known to strongly absorb AFs [33], reducing their bioavailability. At a fortification level of 10,000 µg kg^{-1}, Angle and Wagner [21] observed a slightly but significantly reduced cumulative CO_2 production at the end of 70 days of incubation compared to the control. This is consistent with the present study, in which baseline microbial and fungal respiration began to decrease at the end of incubation at the highest AFB1 concentration. However, these concentrations may be much higher than environmentally relevant levels [1]. Likewise, in the present study, no toxic effects were detected on glucose-induced respiration, a parameter that is much more sensitive to stress, since the provision of the easily decomposable substrate glucose activates a large fraction of the inactive microbes [25]. Moreover, microbial and fungal glucose-induced respiration

increased transiently in the first few days after AFB1 application. One explanation for this increase could be that soil microbes adapted at the cellular level for the purpose of detoxifying AFB1 by producing degradative enzymes. Thus, during AFB1 detoxification, glucose could be co-metabolized alongside with AFB1, leading to an increase in glucose-induced respiration rates. Further investigation through enzyme activity studies of soils exposed to aflatoxins could verify this hypothesis. Therefore, our results suggest that AFB1 at environmentally relevant concentrations does not have a harmful effect on the metabolic activity of the fungi and the overall microbiome.

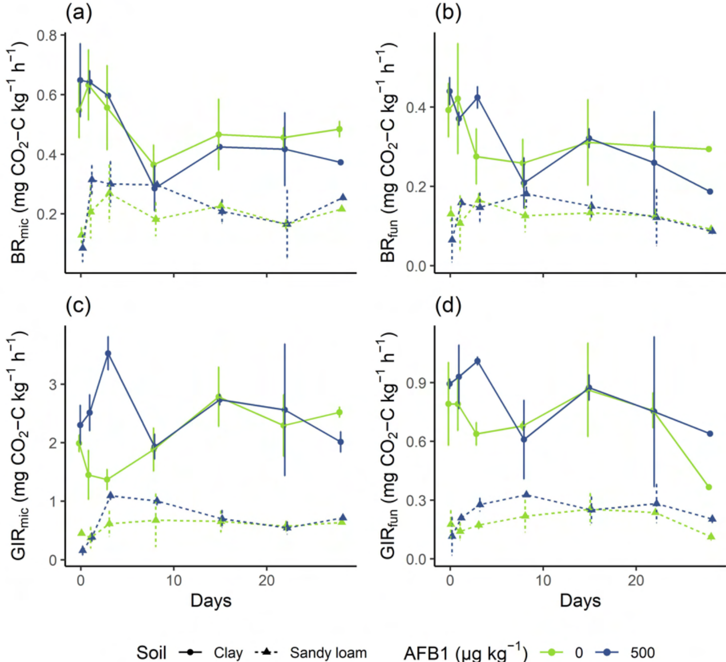

Figure 3. Microbial and fungal activity responses to AFB1 exposure as a function of the incubation time. Curve plots showing mean values for microbial basal respiration BR_{mic} (**a**), microbial glucose-induced respiration GIR_{mic} (**c**), fungal basal respiration BR_{fun} (**b**), and fungal glucose-induced respiration GIR_{fun} (**d**). The error bars represent the standard deviations.

3.3. Carbon Source Utilization Patterns

In clay soil, the overall microbial carbon source utilization in terms of the canonical coefficient significantly increased with AFB1 concentration until the third day (p = 0.03, Appendix D, Table A4, Figure 4). A similar situation was observed for the fungal carbon source utilization in the clay soil, although the increase was not significant (p = 0.296, Appendix D, Table A4, Figure 4). The opposite pattern was observed for the fungal carbon source utilization in the sandy loam soil, where the canonical coefficient decreased from day 1, although not significantly (p = 0.109, Appendix D, Table A4, Figure 4). After the first week, the decrease or increase in the canonical coefficient as a function of AFB1 concentration was less pronounced (Figure 4). Coincidentally, the species weights for all substrates slightly decreased in the sandy loam and slightly increased in the clay soil as compared to the control (Figure 4). In the clay soil, the respiration induced by the readily available carbon substrate glucose (in terms of species weight) was most affected by AFB1 application for both the fungal and whole microbial fungal communities. The respiration induced by all other substrates was much less affected. In sandy soil, the microbial

respiration induced by the amino acid L-alanine, the complex polymer α-cyclodextrin, and glucose was affected by AFB1 application, while for fungal respiration, α-cyclodextrin and glucose-induced respiration were affected (Figure 4).

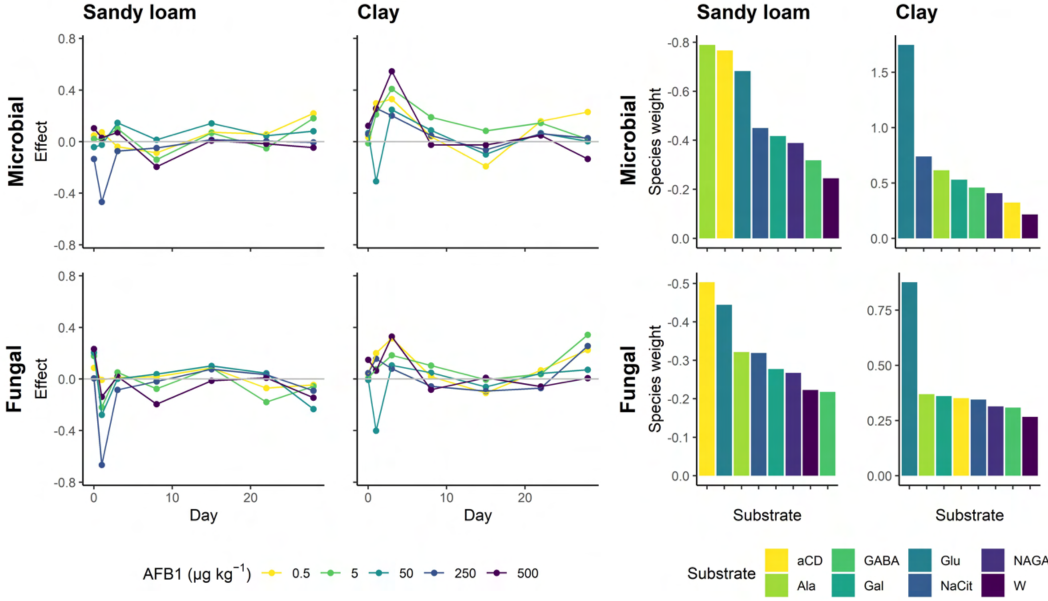

Figure 4. Microbial and fungal carbon source utilization patterns. Left panel: Principal response curves showing the temporal multivariate catabolic response for each AFB1 concentration level as deviations from the nonspiked control (i.e., the zero line). Right panel: Barplots showing the species weights (right) for the response of individual substrates. Glu = D-glucose, Gal = D-galactose, GABA = γ-aminobutyric acid, NAGA = N-acetylglucosamine, NaCit = sodium citrate, and aCD = α-cyclodextrin.

Only minimal negative effects of AFB1 on fungal catabolic profiles occurred in the sandy loam soils. This indicates that overall fungal metabolism in the sandy loam was slightly reduced after AFB1 application. This may be explained by the fast decomposition of AFB1 [1,35,37,39], as reflected in a fast initial drop in extractable AFB1 content in the sandy loam [39]. Furthermore, the particularly complex biopolymer α-cyclodextrin was one of the substrates with the strongest decrease in species weight due to AFB1 exposure. This is in line with our assumption that AFB1-stressed microbial communities are less capable of utilizing more complex carbon substrates. The degradation of complex structures, such as α-cyclodextrin, requires a higher energy investment compared with readily available compounds, since specialized enzymes need to be produced for the decomposition of these polymers [69,70]. Thus, when microbes are exposed to a chemical stressor, they may prefer simple and readily available substrates such as glucose because the energetic gain from utilizing complex substrates such as α-cyclodextrin would not justify the investment required to break down these complex substrates. To gain more comprehensive insights into these processes, enzyme assays targeting different levels of substrate complexity could be conducted. These assays would include enzymes specific to the lignin-degrading system, such as laccase (very complex substrates), polysaccharidases, such as amylases (medium-complexity substrates), and oxidoreductases such as glucose oxidase (readily available substrates). In contrast, the catabolic profile in the clay soil was positively affected until day 3, and the species weights for all substrates were positive. The distinctive pattern

in the first week suggests short-term positive effects of AFB1 on the catabolic profiles of the fungal and whole microbial community in the clay soil. These results are consistent with the increase in microbial biomass carbon observed at the beginning of the incubation experiment (Figure 2). Likewise, the unexpected stimulative effect of AFB1 for the clay soil may be explained by the strong sorption capability of clay minerals. Clay minerals are known to provide sorption sites for dissolved organic compounds (such as the substrates used in this study) [71], as well as soil microbes [72] and their extracellular secreted enzymes [73]. Due to the high sorption affinity of AFB1 to clay minerals, a displacement of these adsorbates from the clay mineral sorption sites may have occurred, as is known for other negatively charged substances such as phosphates [74,75]. A subsequent release of the absorbed substrates, microorganisms, and/or soil enzymes could then have resulted in increased CO_2 production. Another possible explanation for the positive short-term effect of AFB1 on the catabolic response in the clay soil could be stimulation by low available doses of AFB1, resulting in an increased catabolic response. This phenomenon has also been described for secondary metabolites such as alkaloids and is referred to as hormesis [76,77]. Hormesis refers to the beneficial effects of exposure to low doses of a stressor that is typically harmful at higher doses [76]. In the clay soil, the bioavailable AFB1 concentration could be reduced by clay mineral adsorption to be within the hormetic zone, where the metabolic response to low exposure to the chemical stressor is favorable. In the context of aflatoxin exposure, low doses may activate cellular stress response pathways that enhance the microbial ability to deal with subsequent exposure to higher doses of the stressor. Cellular adaption, e.g., the production of enzymes, may lead to the increased co-metabolization of carbon substrates and thus increased CO_2 production. Another explanation could be that AFs in low doses could also be beneficial to microbes by being involved in certain soil reactions themselves. In this context, Finotti, et al. [78] showed that AFs efficiently scavenge peroxides and extend the lifespan of *Escherichia coli* growing under oxidative stress conditions. The authors hypothesized that AFs function as antioxidants and their biological purpose is to extend the lifespan of aflatoxigenic fungi under highly oxidative conditions, such as when substrate resources are depleted. Therefore, the role of AFB1 as a secondary metabolite in further reactions in soils and in terms of microbial responses to stress should be further investigated.

3.4. Soil Microbial and Ecophysiological Ratios

The ERG:C_{mic} significantly decreased with AFB1 concentration in the clay soil ($p < 0.001$) and there was a significant decrease in the ERG:C_{mic} ratio over time ($p = 0.002$, Appendix D, Table A3, Appendix B, Figure A5). The interaction between AFB1 concentration and incubation time was significant ($p = 0.002$, Figure 5a), indicating that the effect of AFB1 was time-dependent. Consistent with the results for C_{mic}, the effect of AFB1 on the ERG:C_{mic} ratio was present only at day 0 and 1, where a strong decrease of about 80% was observed at the highest concentration level (500 µg kg^{-1}) as compared to the control. In contrast, for the sandy loam soil, the ERG:C_{mic} ratio was neither affected by AFB1 concentration ($p = 0.733$) nor by the incubation time ($p = 0.416$). The fungal-to-microbial activity ratios were not affected by AFB1 concentration or by the incubation time (Figure 5b,c).

In the clay soil, both incubation time ($p < 0.001$) and AFB1 concentration ($p = 0.03$) had a significant negative effect on the $Q_{R,mic}$ (Figure 6a), while for the $Q_{R,fun}$ (Figure 6b), no effect of time and AFB1 concentration could be observed (Appendix D, Table A3, Appendix B, Figure A6). In the sandy soil, there were inconsistent effects of AFB1. While the fungal metabolic quotient (Figure 6d) was unaffected by AFB1 concentration ($p = 0.67$), the microbial metabolic quotient (Figure 6c) was slightly but significantly increased by AFB1 ($p = 0.045$, Appendix D, Table A3, Appendix B, Figure A6). Furthermore, there was a significant decrease over time in the metabolic quotient for the soil fungal ($p = 0.031$) and whole microbiome ($p = 0.005$, Appendix D, Table A3, Appendix B, Figure A6).

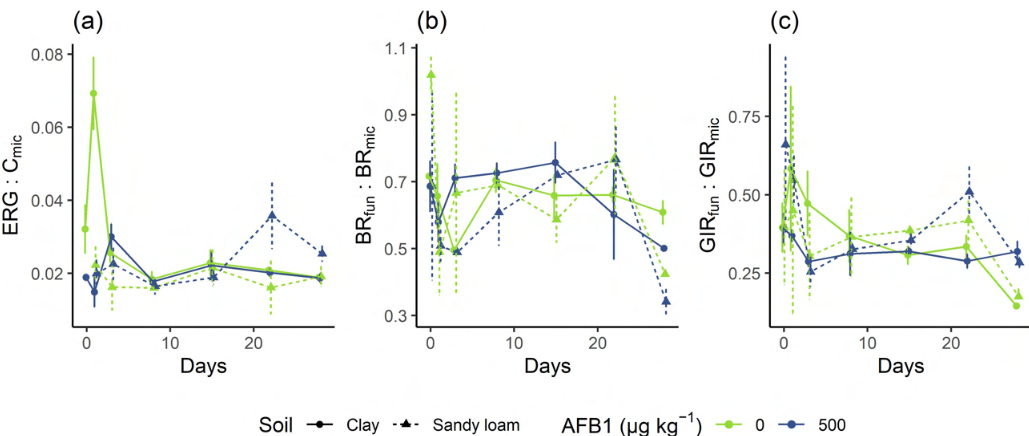

Figure 5. Biomass and activity ratios in AFB1-exposed (500 μg kg^{-1}) and control soil as a function of incubation time. Curve plots showing fungal-to-microbial ratios for the biomass ERG:C_{mic} (**a**), basal respiration BR_{fun}:BR_{mic} (**b**), and glucose-induced respiration GIR_{fun}:GIR_{mic} (**c**).

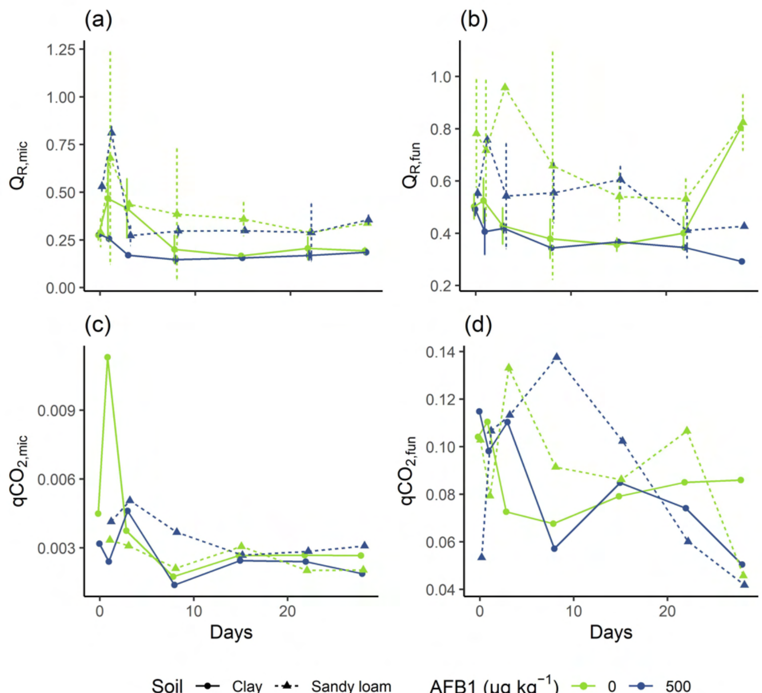

Figure 6. Ecophysiological ratios for AFB1-exposed (500 μg kg^{-1}) and control soils as a function of incubation time. Microbial basal-to-substrate induced respiration $Q_{R,mic}$ (**a**), fungal basal-to-substrate induced respiration $Q_{R,fun}$ (**b**), microbial metabolic quotient $qCO_{2,mic}$ (**c**), and fungal metabolic quotient $qCO_{2,fun}$ (**d**).

For the clay soil, the fungal proportion in terms of ERG:C_{mic} was strongly decreased at the beginning of incubation, in contrast to Burmeister and Hesseltine [19], who observed only limited effects of AFB1 on soil fungal species, while several bacterial species were negatively affected by AFB1. The observed short-term effect on clay soil can also be explained by methodological issues related to the determination of microbial biomass carbon rather than actual changes in soil microbial biomass, as discussed earlier. The strong decrease in the ERG:C_{mic} ratio was driven by a strong increase in the C_{mic} as a function of AFB1 concentration, since the ERG was not significantly affected by AFB1 concentration. For the sandy loam, no effects were observed on the fungal fraction. Likewise, the fungal contribution of the microbial basal and glucose-induced respiration was not affected by AFB1 application. Therefore, it can be assumed that the biomass and the activity of the total microbiome, as well as the soil fungi, were unaffected by AFB1. However, it should be mentioned that the methodology used to detect changes in the activity and structure of the microbial community has a relatively low resolution, as it can only discriminate between effects on fungi and the total microbiome. Methods with a better resolution would allow discrimination even at much lower taxonomic or physiological levels, e.g., quantitative PCR (qPCR) using taxon-specific primers [79] and the analysis of phospholipid fatty acids (PLFA [80,81]).

Regardless of soil, neither the ratio of fungal- nor microbial-induced basal respiration to substrate respiration was increased. Moreover, the microbial $Q_{R,mic}$ was significantly decreased in the clay soil, which was attributable to a significant increase in the GIR, suggesting that a proportion of the potentially active microorganisms were stimulated by AFB1 [25]. As discussed above in relation to the observed increase in catabolic response, the toxicity and/or bioavailability of AFB1 may have been reduced due to sorption to clay minerals, to the extent that a hormetic effect occurred [76]. In contrast, the microbial metabolic quotient was significantly increased in the sandy loam soil as a function of the AFB1 dose at the beginning of incubation, indicating a reduced energetic efficiency in the microbial turnover due to chemical stress [50,51]. The lack of any effect of the fungal basal-to-substrate induced respiration ratio and metabolic quotient suggests that the bacterial fraction of the soil microbiome was mainly affected by AFB1. This is consistent with previous studies. which showed that certain soil bacteria, particularly those that are Gram-positive, are the most affected group [19,20].

4. Conclusions

Aflatoxin B1 has been recognized for its harmful impact on certain bacteria and fungi in in vitro experiments, but its effects on microbial communities in complex environmental systems such as soil have not been systematically investigated. The present study investigated, for the first time, the microbial responses against AFB1 exposure at different physiological levels including biomass, activity, and carbon source utilization patterns, taking into account the complexity of the soil as a matrix. In line with previous studies, it was shown that AFB1 at environmentally relevant concentrations had only minor and transient effects on the biomass and activity of soil microbes. Furthermore, the strength and direction of the observed effects were dependent on the soil. Thus, soil texture largely influenced AFB1 availability. Minor and transitory stimulatory effects on catabolic functionality and microbial activity were observed for clay soil. This suggests that the toxicity and availability of AFB1 was reduced by clay mineral-induced sorption and thus a hormetic effect may have occurred. In contrast, AFB1 in sandy loam soil had a minor negative effect on catabolic functionality and microbial activity, and triggered a slight increase in metabolic quotient. Overall, based on the present study, it can be concluded that AFs do not pose a threat to the integrity of the soil microbiome and thus to soil health for the concentration range and time frame tested. However, although no effects on the community structure in the form of the fungal fraction of the biomass were found, a change in the microbial composition cannot be excluded because the methodology used has only a low taxonomic and physiological resolution. In addition, the present study only investigated the effects of a single AFB1

application on German reference soils, which were presumably never exposed to AFs. Since soils from aflatoxin hotspot regions are frequently exposed to AFs, long-term effects could occur that were not investigated in the present work. Aflatoxin-exposed soils, e.g., from the (sub)tropics in Africa, may be exposed to other stressors such as pesticides, fertilizers, floods, and drought events. The interaction of these stressors with AFs could change the intensity and direction of the effects of AFs on the soil microbiome. Therefore, further studies in the natural environment of aflatoxin-producing fungi are essential to obtain a more comprehensive picture of the environmental relevance of AFs to the soil microbiome and thus soil health.

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Chapter 6

Synthesis and Conclusions

6.1 Challenges in Aflatoxin Analysis and Monitoring in Soil

Understanding the occurrence, fate, and impact of aflatoxins in the soil environment requires a comprehensive and systematic approach that begins with the development of robust analytical methods. This is essential to accurately represent the actual situation of residual aflatoxin concentrations. Four different factors can be considered as major obstacles to the development of analytical methods and sampling strategies: (1) The soil matrix exhibits an inherent complexity characterized by strong interactions between aflatoxins and certain soil fractions (Chapter 1.2.2 and 1.2.4). This phenomenon is similar to that observed in the analysis of various organic pollutants (Trellu et al., 2016). (2) Chromatographic separation is a critical factor in aflatoxin analysis, especially in the context of rapid analysis required for extensive field campaigns. Streamlining the analytical process is essential in such scenarios and requires minimizing or eliminating tedious and costly purification steps while ensuring the effective separation of aflatoxins from co-extracted matrix interferences. (3) The spatial and temporal heterogeneity in the occurrence of mycotoxins in soils further complicates the analysis. This variability is closely related to the various pathways by which aflatoxins can enter the soil environment and the diverse soil processes that determine their fate (Chapter 1.2.1). This heterogeneity is consistent with monitoring campaigns at the food and feed commodity level (Miraglia et al., 2005). (4) Soil is a living matrix in which soil processes such as degradation can play an important role. In this context, appropriate sampling strategies may include recommendations for transport and storage to reliably assess environmental concentrations (Wagner, 1995).

6.1.1 Overcoming Soil–Aflatoxin Interactions in the Extraction of Aflatoxin from Soil

As described in Chapter 1.2, a limited number of studies have investigated the occurrence and biosynthesis, sorption and leaching, and degradation and mineralization of aflatoxins in soil (Accinelli et al., 2008; Goldberg and Angle, 1985; Angle and Wagner, 1980; Angle, 1986). However, interpretation of these results is hampered by insufficient extraction recoveries, the use of spike concentrations well above natural

concentrations, and the lack of systematic validation of analytical methods (Chapter 1.2, Table 1.2).

This obstacle can be attributed to the complicated and heterogeneous nature of the soil as a matrix and the strong adsorption affinity of the AFs at the binding sites in the soil (Chapter 1.2.2). To overcome these methodological challenges, it was essential to disrupt the chemical interactions between the soil matrix and the AFs to facilitate their transition to the liquid phase. The first step, therefore, was to identify the specific soil properties that are primarily responsible for this pronounced interaction and to clarify what underlies these interactions. In this context, as elucidated in Chapter 1.2.2 and 1.2.4, it was found that the strong sorption affinity of AFs to soil can largely be attributed to clay minerals. While studies by Schenzel et al. (2012) and Van Rensburg et al. (2006) demonstrated the interaction of AFs with organic matter, leaching experiments conducted by Goldberg and Angle (1985) on a range of structurally diverse soils underscored that clay content is the main determinant of the particular strong interaction between AF and soil. Kang et al. (2016) further demonstrated that electron donor-acceptor interactions between the two electron-rich carbonyl groups within the coumarin structure of AF and positively charged species located on the negatively charged surfaces of clay minerals (e.g., H^+ for illite and Ca^{2+} for smectite) are primarily responsible for the strong sorption of AFs onto clays.

In this work, the development and validation of a simple and reliable analytical method for the quantification of aflatoxins in soil and plant-based food matrices is described (Chapter 2). The presented approach involved the utilization of an efficient extraction solvent mixture comprising acetonitrile and water, coupled with an ultrasonication step. Recoveries of 78 to 92% were obtained with the presented method, allowing reliable determination at environmentally relevant concentrations of 0.5 to 20 µg kg^{-1}. This is the first time a successful solvent extraction method has been presented for the quantitative analysis of AFs in both soil and food matrices. So far, only one method has achieved a satisfactory recovery of 72%, using the much more complicated and expensive supercritical fluid extraction approach (Starr and Selim, 2008). Acetonitrile, a monopolar solvent with H-bond acceptor properties, exhibited similar characteristics to the carbonyl groups in the coumarin structure

of aflatoxins and consequently displaced the aflatoxins from the H-bond sites on the cations located on the negatively charged surfaces of clay mineral substrates. It is noteworthy that previous studies by Madden and Stahr (1993) using a solvent mixture of similar composition yielded only trace amounts of aflatoxins, probably due to the absence of an ultrasonic step. Ultrasonic treatment is known to reduce the size of soil agglomerates and clay minerals, thereby increasing the surface area (Lesueur et al., 2008). This property makes it a preferred step in the extraction process of organic pollutants from the soil (Bossio et al., 2008). However, the limited selectivity of ultrasonic treatment results in the simultaneous extraction of a high load of matrix components along with the analytes, substantially compromising the analytical performance of the separation and detection method.

6.1.2 Resolving Challenges in Separation and Detection Arising from Matrix Interference

Both LC-MS and HPLC-FLD were found to be suitable for analysis using the method presented in Chapter 2, although there were problems with co-extracted matrix components. Quantitative analysis using MS techniques with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) can be significantly affected by the occurrence of ion suppression or enrichment due to the high ion loading in soil and sediment samples (Trufelli et al., 2010). Therefore, the LC-MS approach experienced signal reductions of up to -25% and -54% for soil and food samples, respectively. Consequently, sample purification techniques such as immunoaffinity chromatography (IAC) or solid-phase extraction (SPE), as well as matrix effect compensation strategies like matrix-matched calibration (MMC) and stable isotope dilution assays (SIDA), would be necessary (Shephard, 2009; Razzazi-Fazeli and Reiter, 2011). In the final procedure presented in Chapter 2, an MMC approach was chosen instead of using costly SIDA or purification steps. However, the use of MMC was only possible because analyte-free samples were available for the matrices under investigation, making the more expensive methods necessary when a sample blank is unavailable.

In contrast, HPLC-FLD exhibited minimal coeluting interferences and negligible matrix effects, rendering it more suitable for routine analysis. To overcome interferences during separation, an unconventional mobile phase composed of a mixture of water, methanol, and acetonitrile (in a ratio of 72:20:8, v/v/v) was employed, offering a compromise between separation efficiency and speed. The relatively high water content of 72% was essential to sufficiently separate interferences from the analytes, albeit at the expense of longer run times. Similarly, a relatively high methanol content was required to achieve an adequate separation between aflatoxins AFG1 and AFB2, leading to extended runtime compared to higher acetonitrile contents. In addition, the HPLC-FLD showed a sensitivity in terms of limit of detection and quantification comparable to LC-MS, which is normally known for its better sensitivity. The sensitivity of the HPLC-FLD was achieved by injecting a high volume of 100 µl, facilitated by the on-column focusing technique (Vissers et al., 1996; Mills et al., 1997; Groskreutz and Weber, 2015) in which the sample was prepared in a weaker solvent (80:20, water/methanol) than the mobile phase (72:20:8, water/methanol/acetonitrile). The absence of a purification step and the ability to use HPLC-FLD significantly reduced the labor and cost of the analytical process. Therefore, this method is particularly promising for routine analysis in regions where aflatoxin levels may be a health concern and require continuous assessment of environmental contamination. In addition, its simplicity and rapidity offer the potential for capacity building, as it does not require complex and expensive analytical equipment. This is particularly beneficial in regions affected by aflatoxin contamination, especially in Sub-Saharan Africa, where lack of advanced analytical equipment and financial constraints can be limiting factors (Gnonlonfin et al., 2012).

6.1.3 Representative Field Sampling in the Face of Spatial Heterogeneity, Seasonality and Aflatoxin Instability

In environmental monitoring of aflatoxins in soil, the challenge is not only to extract aflatoxins from the soil matrix but also to obtain a truly representative soil sample for the entire field or a specific sampling unit. In my thesis (Chapter 3), a comprehensive field study is presented, which aims to investigate the occurrence of AFs in soils and

identify potential influences of agricultural practices, soil depth, and field location. However, no aflatoxins were detected in the soil samples, despite the presence of aflatoxins in maize samples grown in the same field and toxigenic fungi were identified in the soil samples. This inconsistency led to a deeper investigation of the underlying factors.

The inherent heterogeneity of agricultural soils, both in terms of their spatial distribution across fields and their vertical profile, together with the concentrated colonization of grain-rich plant residues by toxigenic fungi (Horn, 2003), may result in localized areas of elevated aflatoxin contamination (Accinelli et al., 2008), with the potential for variation in mycotoxin concentrations even within small regions (Kenngott et al., 2022). To address this small-scale heterogeneity, a sophisticated approach of collecting multiple individual samples from a fine-mesh network of sampling sites within specific sampling clusters at two depths (topsoil and subsoil) and two positions (between plants and inter-row). This methodology had already proven successful in detecting *Fusarium* toxins, including nivalenol and deoxynivalenol, in maize field soils in Germany (Kenngott et al., 2022). Further, the analytical procedure employed for the Kenyan soil extracts adhered closely to the method detailed by Kenngott et al. (2022) and yet yielded negative results for the presence of *Fusarium* toxins, including nivalenol, deoxynivalenol, 15-acetyl-deoxynivalenol, and zearalenone. It's noteworthy that *Fusarium* fungi were indeed detected in these Kenyan soils.

Considering the above factors, it seems unlikely that the absence of aflatoxins in the soil samples was due to an inadequate sampling procedure. Rather, it is plausible that the aflatoxins dissipated during the long storage and transport periods, which spanned approximately 2.5 months from the initial sampling to the analysis phase. This phenomenon was experimentally investigated in Chapter 4 of the study, which revealed that AFB1 was rapidly degraded in two reference soils, with half-lives ranging from 20 to 65 days, depending on various environmental conditions, including UV light, microbial degradation, and sterile conditions.

In summary, the research underscores the importance of frequent timed soil sampling throughout the corn growing cycle in conjunction with analyses immediately

after sampling or proper storage of samples to minimize potential dissipation during transport and storage. These principles align with established practices for monitoring several other soil microbial parameters, including phospholipid-derived fatty acids (Petersen and Klug, 1994; Veum et al., 2019) and soil microbial biomass carbon (Černohlávková et al., 2009; Stenberg et al., 1998), as well as xenobiotics such as pesticides (Lehotay and Cook, 2015). However, it is critical to recognize that these practices extend beyond the laboratory and into international collaborative projects, such as the project conducted with Kenya in this study. As part of such collaborations, capacity building, networking, and the establishment of local laboratory infrastructure are essential. These efforts would enable timely analysis of samples, minimize errors, and ensure accurate results in determining residual concentrations of mycotoxins in the soil environment.

6.2 Environmental Relevance of Aflatoxins in the Soil Environment

When evaluating the environmental relevance of a substance, several aspects must be taken into account, encompassing (1) the extent of the substance's presence in the environment and the factors influencing its occurrence, (2) the environmental fate of the substance in the environment, including the processes it undergoes and the factors influencing these processes, and (3) the consequences of the substance's presence on organisms in the environment and the associated functions. In a review by Fouché et al. (2020), potential ecological consequences associated with aflatoxins occurrence in soil are explored, although there is currently limited empirical evidence available. Furthermore, various reviews (Fouché et al., 2020; Elmholt, 2008; Juraschek et al., 2022) have theoretically elucidated how aflatoxins can enter the soil and how anthropogenic activities may lead to additional aflatoxin inputs, potentially disrupting the balance between depletion and accumulation in soil (Chapter 1.2). However, experimental studies directly investigating the extent and processes of aflatoxin occurrence in soil have been notably scarce. As indicated by Elmholt (2008) and Abbas et al. (2009), one of the primary reasons for this scarcity lies in the unresolved methodological challenges associated with detecting aflatoxins in

soil, which are essential for addressing these research objectives. In this context, the present thesis has successfully addressed some of these methodological issues and provided potential solutions, as detailed in Chapter 6.1. These developments have opened the door to further investigations into the occurrence, fate, and implications of aflatoxins in soil.

6.2.1 Aflatoxin Occurence in Agricultural Soils

Knowledge on the presence of AFs in soils and crop residues remains limited, and little information is available on the extent and causes. A notable contribution in this field was made by Accinelli et al. (2008), who demonstrated that aflatoxins are synthesized in the soil at varying levels i.e. in the range of 10^2 (cobs containing grain), 10^0 (leaves, stalks and cobs without grain) and 10^{-1} μg kg^{-1} (soil). In addition, they demonstrated that although AFB1 appears to be transient in soil, it is apparently produced in surface soil in the presence of corn residues. This production was evidenced by *A. flavus* CFU levels, detection of AFB1 in soil, and expression of genes related to aflatoxin biosynthesis. This is consistent with the results of this thesis, in which no aflatoxins were detected in soils from high-risk areas in Kenya, although samples were tested for soil fungi capable of producing aflatoxins (Chapter 3).

The factors and agricultural practices that influence the occurrence of aflatoxins in crops at the preharvest stage have already been studied (see Chapter 3). However, the influence of these factors on the occurrence of aflatoxins in soil remains largely unexplored. To bridge this knowledge gap, a large-scale field study was conducted in Chapter 3 within a high-risk model region for aflatoxin contamination in Sub-Saharan Africa, namely the Makueni region in Kenya. The objective of this study was to investigate the occurrence of aflatoxins in soils while identifying potential influences of agricultural practices, soil depth, and field location. Interestingly, no aflatoxins were detected in the soil samples. From these results, particularly the absence of aflatoxins in the soil of a model region at high risk for aflatoxin contamination, it could be concluded that aflatoxins are not present in soil at environmentally relevant levels. However, several factors challenge this conclusion. Notably, the absence of aflatoxins is likely due to degradation to undetectable levels during the 2.5-month

transport (Chapter 3). Additionally, the occurrence of aflatoxins in the soil may be subject to a seasonal cycle. Given that aflatoxin-producing fungi were identified in the soil samples, it is plausible that *in situ* production occurs during the early stages of crop cultivation, particularly when soil moisture recovers, leading to the germination of *Aspergillus* sclerotia and spores, followed by the growth of the fungus (Accinelli et al., 2008; Elmholt, 2008). Furthermore, heavily contaminated plant material, unsuitable for commercialization, is frequently incorporated into the soil post-harvest (Horn, 2003; Horn et al., 1995), potentially representing a period of elevated aflatoxin concentration in the soil.

In conclusion, this work has revealed uncertainties regarding the extent of aflatoxin contamination in soil. Future research efforts should aim to investigate the temporal dynamics of aflatoxin occurrence in soil and explore the potential for in situ production by aflatoxin-producing fungi during the early stages of crop cultivation.

6.2.2 Dissipation of Aflatoxins in Soil Systems

In the context of assessing the environmental relevance of a substance, understanding its persistence in the environment is critical since the rate of dissipation has a central function in determining the duration and intensity of potential ecological effects. Soil dissipation processes result from a combination of microbial, physical, and chemical factors. Previous literature, as reviewed in Chapter 1.2, suggested that aflatoxins in soil are rapidly degraded, with half-lives ranging from days to weeks, and that microbial degradation is the predominant dissipation process (Accinelli et al., 2008; Angle and Wagner, 1980; Angle, 1986). In contrast, abiotic degradation processes in soil are generally considered negligible (Fouché et al., 2020), an assertion that lacks empirical support, as only one experimental study has examined abiotic degradation in soil so far (Accinelli et al., 2008). However, given the short half-lives of aflatoxins under exposure to physical and chemical conditions such as UV light, organic acids, and ammonia, it is plausible that (photo)chemical degradation could contribute significantly to aflatoxin degradation in soil. Moreover, the interplay between microbial and (photo)chemical degradation processes in relation to available AFB1 concentration and soil physicochemical properties is still largely unexplored.

To address these knowledge gaps, Chapter 4 presents a controlled laboratory experiment to systematically investigate the degradation of AFB1 in soil considering microbial, photochemical, and dark abiotic conditions in two different soil types (sandy loam and clay soil) and at varying initial AFB1 concentrations. Results showed AFB1 dissipation and AFB2a formation occurred in all soils and conditions. Notably, photochemical degradation emerged as a major degradation process, alongside the well documented predominance of microbial degradation. However, it should be noted that photodegradation is likely limited to AF contaminated material at the soil surface and in the topsoil due to the high light attenuation potential of the soil. Moreover, the determined half-lives of microbial degradation were considerably longer than previous studies, possibly due to drier conditions (40% WHC) compared to earlier research with 80 - 100% WHC (Accinelli et al., 2008; Angle and Wagner, 1980; Angle, 1986). These findings suggest previous studies may have underestimated aflatoxin persistence in soil, particularly especially in drier conditions, such as those found in subtropical regions. In the sandy loam soil, higher initial AFB1 concentrations correlated with slower dissipation rates, likely due to toxic effects on microorganisms. This trend was absent in clay soil, probably due to reduced bioavailability by AFs sorption onto clay minerals. In all degradation scenarios, only AFB2a was detected as a transformation product, which is consistent with the findings of Starr et al. (2017), who argued that the presence of the metabolites AFB2, AFG1, and AFG2 reported in previous studies (Angle and Wagner, 1980; Angle, 1986) resulted from misidentification, primarily attributable to the use of thin-layer chromatography. However, the amount of AFB2a formed did not account for the total dissipated AFB1. Mass balance analysis suggested a significant portion of dissipated AFB1 in a non-quantifiable fraction, whose exact nature remains unclear, whether it involves volatilization, mineralization to CO_2, bound residues, or incorporation into microbial biomass. Further investigations, such as radiotracer analysis, are needed to clarify this.

In conclusion, my thesis underscored the significance of different degradation processes in determining the fate of aflatoxins in soil. For the first time, it was demonstrated that, alongside microbial degradation, (photo)chemical degradation

can be a significant detoxification process, and that these processes are modulated by soil properties and initial aflatoxin concentration. These results contribute to the understanding of aflatoxins as micropollutants in the soil and highlight the role of soil properties in AFB1 degradation processes. Nevertheless, questions regarding the non-quantifiable contribution and the nonlinear effect of initial concentration on microbial degradation in clay soils remain unanswered, motivating further research.

6.2.3 Soil Environmental Implications of Aflatoxin Exposure

As outlined in Chapter 1.2, there is substantial evidence indicating that aflatoxins exert toxic effects on certain soil microorganisms. One plausible explanation for this phenomenon is that aflatoxins may be produced as a protective response to microbial competition or predation (Elmholt, 2008). However, it should be noted that conflicting results exist in this regard, with some studies reporting toxic effects while others do not (Burmeister and Hesseltine, 1966; Arai et al., 1967; Angle and Wagner, 1981). Critically, the majority of these effect studies were conducted under optimized *in vitro* conditions, typically involving cultivation on agar media that do not consider soil as a natural environmental matrix (Drott et al., 2019). Moreover, these studies often focused solely on assessing the effects on microbial biomass, growth, and activity. This approach presents several limitations: (1) It excludes the influence of natural external factors to which these organisms may be exposed in the environment, factors that could significantly influence the magnitude and direction of the observed effects; (2) less than 1% of the total microbiome can be cultured on agar media (Pham and Kim, 2012), rendering the results non-representative of the entire microbiome; (3) it fails to assess the impact on the physiology and functionality of the microbiome, even though these aspects are crucially linked to soil functions. Current methods of disposing of crops contaminated with AFs, which often involve their incorporation into the soil, could result in elevated natural contamination levels and potential disruption of the ecological balance (Fouché et al., 2020). This emphasizes the need for a comprehensive approach to gain a full understanding of the ecological function of AFs and to assess their potential impact on soil health. This should consider soil

as a complex heterogeneous environmental matrix and examine microbial responses at different physiological levels.

To address this research gap, Chapter 5 presents a laboratory study that examined soil microbial responses to AF exposure across a range of environmentally relevant concentrations, focusing on multiple physiological response levels, including biomass, activity, carbon source utilization patterns and ecophysiological ratios, thereby considering soil as a complex heterogeneous environmental matrix. Consistent with previous studies, it was shown that AFB1 at environmentally relevant concentrations had only minor and transient effects on soil microbial biomass and activity. Furthermore, the magnitude and direction of these observed effects depended on the soil type. Soil texture particularly affected AFB1 availability, which is consistent with observations on microbial and (photo)chemical degradation (Chapter 4). In clay soils, minor and transient stimulatory effects on catabolic functionality and microbial activity were observed, suggesting that AFB1 toxicity and availability were reduced by clay mineral-induced sorption, eventually leading to hormetic effects. This observation could also explain the nonlinear effect of initial concentration on microbial degradation in clay soils (Chapter 4). In contrast, sandy loam soils showed minor negative effects on catabolic functionality and microbial activity in response to AFB1 exposure, along with a slight increase in metabolic quotient.

In summary, it can be concluded on the basis of this thesis that aflatoxins do not pose a threat to the integrity of the soil microbiome and thus to soil health within the concentration range and time frame investigated. This is particularly true for clayey soils, where the toxicity of AFs is significantly reduced due to their strong binding to clay minerals. This relationship is consistent with research in various fields, including livestock, where clay minerals are used as binders in animal feed to reduce the uptake of aflatoxins by animals and thus mitigate potential harmful effects (Jaynes et al., 2007; Wan et al., 2013; Schell et al., 1993). Therefore, these results highlight the critical role of considering soil structure, particularly clay content, in assessing the environmental impact of aflatoxins on the soil microbiome. Nevertheless, it is important to point out some limitations. No effects on community structure, particularly on the proportion of fungi in the biomass, were detected. However, changes in microbial composition

cannot be excluded because the methodology used had limited taxonomic and physiological resolution. In addition, this study only examined the effects of a single AFB1 application event on German reference soils, which are assumed to have never been exposed to AFs. Soils in regions affected by aflatoxins, such as the (sub)tropical areas of Africa, are likely to be regularly contaminated with AF, which may lead to repeated exposure with unexplored longterm effects. In addition, these aflatoxin-impacted soils may face several stressors, including pesticides, fertilizer overuse, floods, and droughts. The interaction between these stressors and aflatoxins could change the magnitude and direction of the impact of aflatoxins on the soil microbiome and thus could impair soil health. Overall, this indicates that further research in the natural habitats of aflatoxin-producing fungi is needed to gain a more comprehensive understanding of the ecological importance of AFs to the soil microbiome and thus to soil health.

6.3 Conclusion and Future Aspects

The main objective of this dissertation project was to investigate the environmental relevance of aflatoxins in soil by scrutinizing the mechanisms and extent of aflatoxin occurrence in soil, the processes of their dissipation and their effects on the soil microbiome and associated soil functions, with regard to soil properties. Several methodological challenges that had previously hindered the investigation of the environmental relevance of aflatoxins in soil were successfully overcome. In particular, the development of a reliable and cost-effective analytical procedure has paved the way for aflatoxin research in the soil environment. Importantly, this method was designed with minimal cost and labor, making it applicable in resource-limited regions, particularly in subtropical areas where aflatoxin problems are widespread. A large-scale field trial was conducted with the aim of detecting aflatoxins in field soil and evaluating the influence of factors such as location, depth, soil properties and agricultural practices. The fact that no aflatoxins were detectable in this study highlighted that monitoring in the field remains challenging. These challenges include rapid degradation, spatial heterogeneity, and seasonality of aflatoxin occurrence,

which must be considered in future field studies. Furthermore, this research has shown that aflatoxins undergo rapid dissipation in soil, highlighting the importance of abiotic degradation mechanisms, especially photolytic degradation, in the detoxification of aflatoxins in the soil. The influence of soil characteristics, particularly texture, on these processes has been underscored. Nevertheless, the causes of the dissipation of aflatoxins in soil remain uncertain and require further investigation in future studies. The study of the effects of aflatoxins on the soil microbiome and soil functions has shown that aflatoxins do not pose a significant threat to soil health, especially in clayey soils.

However, important questions remain unanswered, highlighting the need for further research to gain a more complete understanding of the ecological significance of aflatoxins. Looking ahead, future research should focus on addressing the challenges of field monitoring of aflatoxins, elucidating the mechanisms underlying the dissipation processes of aflatoxins in the soil during microbial and (photo)chemical degradation scenarios, further investigating the ecological consequences of aflatoxins, especially in regions that are severely affected by aflatoxin issues, and exploring the complex interactions between aflatoxins and various environmental and anthropogenic stressors. By answering these questions, we can increase our knowledge of the environmental impact of aflatoxins on soil health and ultimately contribute to more effective strategies for managing aflatoxins in agriculture.

Chapter 7

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Chapter 8

Annex

8.1 Supporting Information on Chapter 1

Table 8.1: Occurrence of AFB1, AFM1 and the sum of the aflatoxins (AFB1 + AFB2 + AFG1 + AFG2) in food products from countries across different geographical regions.

Geographical region	Country	Food product	Aflatoxin	N	% pos.	Range [a]	References
Central America	Costa Rica	Milk	AFM1	70	95.7	0.019–0.629	Chavarría et al. (2015)
		Cheese		70	37.2	0.031-0.276	
	Haiti	Peanut	ΣAFs	8	25	186.6–375.1	Aristil et al. (2020)
	Mexico	Infant formula	AFM1	55	20	0.040–0.450	Quevedo-Garza et al. (2020)
East Asia	China	Cow milk	AFM1	5650	4.7	0.0085–0.412	Li et al. (2017)
	China	Cow milk	AFM1	233	48.1	0.005–0.096	Guo et al. (2013)
		Yoghurt		178	4.5	0.005–0.083	
	China	Peanuts	ΣAFs	2494	0.15	0.06–1602.5	Wu et al. (2016)
	China	Raw milk	AFM1	1207	4.64	0.005–0.06	Li et al. (2018)
	China	Rice	AFB1	370	63.5	0.03–20	Lai et al. (2015)
	China	Wheat & wheat crackers	AFB1	178	5.6	0.03–0.12	Zhao et al. (2017)
	Korea	Functional foods	AFB1	185	0	ND	Lee et al. (2015)
	Korea	Brown rice	AFB1	507	1	0.3–1.1	Kim et al. (2017)
		Millet			9	0.4–5.6	
		Sorghum			4	0.7–1.7	
		Maize			1	0.7–5.2	
		Mixed cereals			4	0.4–12.4	
	Korea	Soybean paste	ΣAFs	45	24.4	0.88–16.17	Jeong et al. (2019)
	Taiwan	Peanut products	ΣAFs	1827	32.7	0.2–513.4	Chen et al. (2013)
	Taiwan	Peanuts	AFB1	1089	25	0.2–432	Lien et al. (2019)

Table 8.1 continued from previous page

Geographical region	Country	Food product	Aflatoxin	Sample size	% positive	Range [a]	References
			AFB2			0.1–130.9	
			AFG1			0.2–113	
			AFG2			0.1–17	
			ΣAFs			0.1–441	
Middle East/	Egypt	Maize	AFB1	61	25	0.02–44.9	Abdallah et al. (2019)
North Africa			AFB2	10	0.1-7.0		
		Milk	AFM1	20	0.02-0.19		
	Egypt	Meat products	ΣAFs	50	100	0.47–2.1	Abd-Elghany and Sallam (2015)
	Egypt	Wheat	AFB1	36	33.3	0.04–62.17	Hathout et al. (2020)
			AFB2	75	0.12-3.82		
			AFG1	100	0.09-48.59		
			AFG2	100	0.11-10.93		
	Iran	Cow milk	AFM1	64	84.4	0.006–0.188	Bahrami et al. (2016)
		Yoghurt		42	23.8	0.006–0.021	
	Iran	Rice	AFB1	40	100	0.29–2.92	Eslami et al. (2015)
	Iran	Wheat flour	ΣAFs	180	80	0.01–0.5	Jahanbakhsh et al. (2019)
	Lebanon	Infant formula	AFM1	84	88	0.005–0.0481	Elaridi et al. (2019)
	Lebanon	Spices	AFB1	94	16	2.2–1118.3	El Darra et al. (2019)
		Herbs		38	8	8.7–62.7	
	Morocco	Tea	ΣAFs	1290	58.9	1.2–116.2	Mannani et al. (2020)
	Saudi Arabia	Nuts	ΣAFs	264	26.5	1.0–110	El Tawila et al. (2013)
	Tunisia	Pearl Millet	AFB1	220	8.6	0.24–1046	Houissa et al. (2019)
			AFB2		0.5	0.4–96.1	

Table 8.1 continued from previous page

Geographical region	Country	Food product	Aflatoxin	Sample size	% positive	Range [a]	References
			AFG1		0.5	0.32–20.3	
			AFM1		0.5	0.4–18.1	
	Yemen	Roasted coffee beans	ΣAFs	25	100	14.255–23.231	Humaid et al. (2019)
		Green coffee beans		25	100	14.694–27.176	
North America	USA	Chilies	AFB1	169	63.9	2–94.9	Singh and Cotty (2017)
South America	Brazil	Cashew nuts	ΣAFs	70	34.3	0.60–31.5	Milhome et al. (2014)
	Brazil	Cocoa beans	ΣAFs	123	16.3	0.35–30	Pires et al. (2019)
	Brazil	Cow milk	AFM1	129	14	0.0002–0.1057	Picinin et al. (2013)
	Brazil	Maize	ΣAFs	148	38	0.5–49.9	Oliveira et al. (2017)
	Brazil	Peanuts	ΣAFs	119	10	0.3–100	Martins et al. (2017)
	Colombia	Maize	AFB1	20	15	6.4–458.2	Diaz et al. (2015)
			AFB2		15	1.9–55.5	
			AFG1		5	72.2–72.2	
	Peru	Maize	ΣAFs	82	64.6	1–17	Coloma et al. (2019)
	Uruguay	Sorghum	AFB1	275	0.7	1–14	Del Palacio et al. (2016)
South Asia	India	Corn	AFB1	150	100	48–383	Mudili et al. (2014)
	India	Rice	ΣAFs	87	2.3	21.581–22.989	Mukherjee et al. (2019)
	India	Sorghum	AFB1	15	71.4	0.005–0.02	Jayashree and Wesely (2019)
	India	Spices	ΣAFs	55	85.4	4–219.6	Jeswal and Kumar (2015)
	Pakistan	Dates & dates products	ΣAFs	57	31.6	0.15–16.70	Iqbal et al. (2014)
	Pakistan	Milk	AFM 1	520	93.1	0.001–0.26	Ismail et al. (2016)
	Pakistan	Raw milk	AFM1	960	70	0.3–1.0	Akbar et al. (2019)
	Pakistan	Rice	AFB1	2047	73.3	1.17–6.91	Asghar et al. (2016)

Table 8.1 continued from previous page

Geographical region	Country	Food product	Aflatoxin	Sample size	% positive	Range [a]	References
	Pakistan	Rice & rice products	AFB1	208	35.1	0.04–21.3	Iqbal et al. (2016)
			ΣAFs		35.1	0.04–32.2	
	Pakistan	Tea	ΣAFs	94	78.3	0.11–16.17	Ismail et al. (2020)
Southeast Asia	Malaysia	Cow milk	AFM1	102	2	0.020–0.142	Shuib et al. (2017)
	Malaysia	Milk & milk products	AFM1	53	35.8	0.0035–0.1005	Nadira et al. (2017)
	Malaysia	Spices	ΣAFs	34	85	0.01–9.34	Ali et al. (2015)
	Vietnam	Maize	AFB1	2370	33.7	0.02–34.8	Lee et al. (2017)
Southern Europe	Greece	Milk	AFM 1	196	46.4	0.005–0.016	Tsakiris et al. (2013)
	Greece	Sesame seeds	AFB1	30	77.6	0.02–14.49	Kollia et al. (2016)
	Italy	Cow milk	AFM1	416	12.3	0.004–0.052	De Roma et al. (2017)
		Buffalo milk		388	7.2	0.004–0.031	
	Italy	Spices	AFB1	130	15.4	0.59–5.38	Prelle et al. (2014)
	Kosovo	Raw milk	AFM1	826	2.8	0.005–0.05	Rama et al. (2016)
		UHT milk		69	2.6	0.005–0.05	
	Macedonia	Raw milk	AFM1	3635	42.4	0.0066–0.4081	Dimitrieska-Stojković et al. (2016)
	Portugal	Milk	AFM1	40	27.5	0.005–0.069	Duarte et al. (2013)
	Serbia	Corn	ΣAFs	380	36.1	1.01–86.1	Kos et al. (2013)
	Serbia	Milk	AFM 1	176	93.8	0.01–1.20	Kos et al. (2014)
	Serbia	Maize	AFB1	56	48.2	0.04–8.80	Torović (2017)
			ΣAFs		48.2	0.04–9.14	
	Serbia	Milk	AFM1	80	92.5	0.003–0.319	Torović (2015)
		Infant formula		21	4.8	0.03–0.02	
	Serbia	Raw milk	AFM1	678	100	0.025–>1	Tomašević et al. (2015)

Table 8.1 continued from previous page

Geographical region	Country	Food product	Aflatoxin	Sample size	% positive	Range [a]	References
		Heat treated milk		438	100	0.025–1	
		Milk products		322	100	0.025–>1	
	Spain	Cereals	ΣAFs	67	0	ND	Vidal et al. (2013)
	Spain	Toasted cereal flour	AFB1	94	25.5	0.025–0.17	Luzardo et al. (2016)
			AFB2		24.5	0.025–0.07	
			AFG1		9.6	0.025–0.12	
			AFG2		8.5	0.025–0.17	
	Spain	Wheat (pizza dough)	AFB1	60	23	1.03–9.50	Quiles et al. (2016)
			AFB2		32	0.34–0.67	
	Turkey	Cow milk	AFM1	176	30.1	0.025–1.01	Golge (2014)
	Turkey	Hazelnuts	ΣAFs	170	6.5	0.09–11.3	Kabak (2016)
		Dried figs		130	12.3	0.1–28.2	
	Turkey	Maize	ΣAFs	1055	4	7.96–163.62	Artik et al. (2016)
	Turkey	Peanut	ΣAFs	102	84	0.2–2177.2	Meena et al. (2019)
	Turkey	Wheat	ΣAFs	141	2	0.21–0.44	Turksoy and Kabak (2020)
	Turkey	Wheat flour	AFB1	60	0	ND	Kara et al. (2015)
		Maize flour		24	66.7	0.041–1.12	
Sub Saharan Africa	Burkina Faso	Sorghum malt	AFB1	50	25	46.33–254.73	Bationo et al. (2015)
	Congo	Corn (pre-harvest)	ΣAFs	50	32	3.1–103.89	Kamika et al. (2016)
		Corn (post harvest)		150	52	1.5–2806.5	
	Ethiopia	Groundnuts	ΣAFs	120	77.5	15–11900	Chala et al. (2013)
	Ethiopia	Maize	ΣAFs	150	100	20–91.04	Chauhan et al. (2016)
	Ethiopia	Peanut	AFB1	160	26.9	1.0–2526	Mohammed et al. (2016)

Table 8.1 continued from previous page

Geographical region	Country	Food product	Aflatoxin	Sample size	% positive	Range [a]	References
			AFB2		27.5	0.05–237	
			AFG1		5.3	1–736	
			AFG2		5.5	0.05–171	
	Ethiopia	Sorghum	AFB1	90	100	1–33.10	Taye et al. (2016)
	Ghana	Maize	ΣAFs	326	37.7	0.1–341	Agbetiameh et al. (2018)
	Kenya	Pearl Millet	AFB1	205	64	1.0–1658.2	Sirma et al. (2016)
	Kenya	Raw milk	AFM1	96	100	0.0154–4.563	Kuboka et al. (2019)
	Malawi	Nut-based foods	AFB1	55	78.2	0.1–40.6/6.28	Matumba et al. (2014)
	Namibia	Sorghum malt	AFB1	45	44	0.61–28.3	Nafuka et al. (2019)
			AFB2		9	0.14–2.35	
			AFG1		17	0.39–6.95	
	Nigeria	Chilies	AFB1	55	38.2	2–156	Singh and Cotty (2017)
	Nigeria	Ginger	AFB1	120	55	0.11–8.76	Lippolis et al. (2017)
			AFB2		36.7	0.13–1.01	
			ΣAFs		55	0.11–9.52	
	Nigeria	Peanut	AFB1	84	29.8	0.9–710	Oyedele et al. (2017)
			AFB2		17.9	0.4–129	
			AFG1		22.6	0.4–1202	
			AFG2		7.1	18.3–123	
			ΣAFs		39.3	0.4–2076	
	Nigeria	Rice	AFB1	38	18.4	0.15–20.2	Rofiat et al. (2015)
			AFB2		13.2	0.2–6.11	
			AFG1		5.3	0.2–7.21	

Table 8.1 continued from previous page

Geographical region	Country	Food product	Aflatoxin	Sample size	% positive	Range [a]	References
	Nigeria	Roasted cashew nuts	ΣAFs	27	100	0.1–6.8	Adetunji et al. (2018)
	Nigeria	Sorghum	ΣAFs	146	28.6	0.96–21.74	Daneil et al. (2016)
	Togo	Maize	AFB1	70	76	1.1–75.9	Hanvi et al. (2021)
	Uganda	Maize	ΣAFs	256	25.8	0–3760	Sserumaga et al. (2020)
	Zambia	Peanuts	AFB1	92	44.6	0.015–46.60	Bumbangi et al. (2016)
			ΣAFs		55.4	0.014–48.67	
	Zimbabwe	Corn	AFB1	388	20.6	0.75–26.6	Murashiki et al. (2017)

ND: Not detected

[a] Aflatoxin concentrations are expressed in µg kg $^{-1}$ for solid matrices and µg L $^{-1}$ for liquid matrices.

National and Internationally Harmonized Limits for Aflatoxins in Foodstuffs

To gain an overview of the changes in the legal limits for aflatoxins in food, both at the national and international level, over a period of 20 years, a comparative analysis was carried out between the years 2002 and 2022. The data research revealed that in most countries, regulatory limits have been established for the sum of the four major aflatoxins (AFB1, AFB2, AFG1, and AFG2) and/or for the most toxic aflatoxin (AFB1), particularly in the context of maize and peanuts. Consequently, data were collected for these specific foods and parameters. In 2002, the Food and Agriculture Organization of the United Nations (FAO) made an important contribution in this area by conducting a comprehensive study aimed at assessing the global landscape of mycotoxin regulation (Van Egmond and Jonker, 2004). This study found that for corn and peanuts, a total of 89 countries have set limits. Of these, 67 countries set national standards, while 22 countries adopted internationally harmonized standards within economic unions such as the European Union (EU), Common Market of the South (Mercosur) and Australia/New Zealand. In the following years, more countries set their own national standards, and more nations joined these economic unions, e.g. through the eastward expansion of the EU. In addition, the emergence of economic unions such as ARSO (African Organization for Standardization), EAC (East African Community), EACU (Eurasian Customs Union) and GSO (Gulf Cooperation Council Standardization Organization) contributed to the implementation of limits by more countries. The following table shows the status of regulation at the national and international level for different countries and time periods. This data was then used for creating world maps (Figure 1.4). In cases where multiple limits existed for a given time period and nation, the most stringent value was used for visualization.

Table 8.2: The situation of aflatoxin regulation in the year 2002 and 2022: Regulation limits for AFB1 and the sum of AFB1, AFB2, AFG1, and AFG2 in maize and peanuts, considering both countries that follow internationally harmonized aflatoxin standards and those that set their own national limits.

Country	Hierarchy	Economic union	Time stamp	Food	AFB1[a]	ΣAFs[a]	Reference
Algeria	National	-	2002	Maize	10	20	Van Egmond and Jonker (2004)
Algeria	International	ARSO	2022	Maize	5	10	ARSO (2022)
Algeria	National	-	2002	Peanut	10	20	Van Egmond and Jonker (2004)
Algeria	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Argentina	International	Mercosur	2002	Maize	NA	20	Mercosur (2002)
Argentina	International	Mercosur	2002	Peanut	NA	20	Mercosur (2002)
Armenia	International	EACU	2022	Maize	5	NA	EACU (2011)
Armenia	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Armenia	International	EACU	2022	Peanut	5	NA	EACU (2011)
Armenia	National	-	2002	Maize	5	NA	Van Egmond and Jonker (2004)
Australia	International	AU&NZ	2002	Peanut	NA	15	FSANZ (2022)
Austria	International	EU	2002	Maize	2	4	EC (2010)
Austria	International	EU	2022	Maize	2	4	EC (2010)
Austria	International	EU	2002	Peanut	2	4	EC (2010)
Austria	International	EU	2022	Peanut	2	4	EC (2010)
Bahrain	International	-	2022	Maize	NA	4	Van Egmond and Jonker (2004)
Bahrain	International	-	2022	Peanut	NA	4	Van Egmond and Jonker (2004)
Bangladesh	National	-	2022	Peanut	NA	10	BFSA (2017)
Barbados	National	-	2002	Maize	NA	20	Van Egmond and Jonker (2004)
Barbados	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Belarus	International	EACU	2022	Maize	5	NA	EACU (2011)
Belarus	International	EACU	2022	Peanut	5	NA	EACU (2011)
Belgium	International	EU	2002	Maize	2	4	EC (2010)
Belgium	International	EU	2022	Maize	2	4	EC (2010)
Belgium	International	EU	2002	Peanut	2	4	EC (2010)
Belgium	International	EU	2022	Peanut	2	4	EC (2010)
Belize	National	-	2002	Maize	NA	20	Van Egmond and Jonker (2004)
Belize	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Benin	International	ARSO	2022	Maize	5	10	ARSO (2022)
Benin	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Botswana	International	ARSO	2022	Maize	5	10	ARSO (2022)
Botswana	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Brazil	International	Mercosur	2002	Maize	NA	20	Mercosur (2002)
Brazil	International	Mercosur	2002	Peanut	NA	20	Mercosur (2002)
Bulgaria	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Bulgaria	International	EU	2022	Maize	2	4	EC (2010)
Bulgaria	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Bulgaria	International	EU	2022	Peanut	2	4	EC (2010)
Burkina Faso	International	ARSO	2022	Maize	5	10	ARSO (2022)
Burkina Faso	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Burundi	International	ARSO	2022	Maize	5	10	ARSO (2022)
Burundi	International	EAC	2022	Maize	5	10	EAC (2018)
Burundi	International	ARSO	2022	Peanut	5	10	ARSO (2022)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Burundi	International	EAC	2022	Peanut	5	10	EAC (2018)
Cameroon	International	ARSO	2022	Maize	5	10	ARSO (2022)
Cameroon	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Canada	National	-	2002	Peanut	NA	15	Van Egmond and Jonker (2004)
Chad	International	ARSO	2022	Maize	5	10	ARSO (2022)
Chad	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Chile	National	-	2002	Maize	NA	5	Van Egmond and Jonker (2004)
Chile	National	-	2002	Peanut	NA	5	Van Egmond and Jonker (2004)
China	National	-	2002	Maize	20	NA	Van Egmond and Jonker (2004)
Colombia	National	-	2002	Maize	NA	20	Van Egmond and Jonker (2004)
Colombia	National	-	2002	Peanut	NA	10	Van Egmond and Jonker (2004)
Costa Rica	National	-	2002	Maize	NA	35	Van Egmond and Jonker (2004)
Costa Rica	National	-	2022	Maize	NA	20	MHCR (2011b)
Costa Rica	National	-	2022	Peanut	NA	15	MHCR (2011a)
Croatia	International	EU	2002	Maize	2	4	EC (2010)
Croatia	National	-	2002	Maize	5	NA	Van Egmond and Jonker (2004)
Croatia	International	EU	2022	Maize	2	4	EC (2010)
Croatia	International	EU	2002	Peanut	2	4	EC (2010)
Croatia	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Croatia	International	EU	2022	Peanut	2	4	EC (2010)
Cuba	National	-	2002	Maize	5	5	Van Egmond and Jonker (2004)
Cuba	National	-	2002	Peanut	5	5	Van Egmond and Jonker (2004)
Cyprus	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Cyprus	International	EU	2022	Maize	2	4	EC (2010)
Cyprus	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Cyprus	International	EU	2022	Peanut	2	4	EC (2010)
Czech Republic	International	EU	2022	Maize	2	4	EC (2010)
Czech Republic	International	EU	2022	Peanut	2	4	EC (2010)
CzechRepublic	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
CzechRepublic	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Democratic Republic of the Congo	International	ARSO	2022	Maize	5	10	ARSO (2022)
Democratic Republic of the Congo	International	EAC	2022	Maize	5	10	EAC (2018)
Democratic Republic of the Congo	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Democratic Republic of the Congo	International	EAC	2022	Peanut	5	10	EAC (2018)
Denmark	International	EU	2002	Maize	2	4	EC (2010)
Denmark	International	EU	2022	Maize	2	4	EC (2010)
Denmark	International	EU	2002	Peanut	2	4	EC (2010)
Denmark	International	EU	2022	Peanut	2	4	EC (2010)
Djibouti	International	ARSO	2022	Maize	5	10	ARSO (2022)
Djibouti	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Ecuador	National	-	2002	Maize	10	20	Van Egmond and Jonker (2004)
Ecuador	National	-	2002	Peanut	5	10	Van Egmond and Jonker (2004)
Egypt	International	ARSO	2022	Maize	5	10	ARSO (2022)
Egypt	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Estonia	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Estonia	International	EU	2022	Maize	2	4	EC (2010)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Estonia	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Estonia	International	EU	2022	Peanut	2	4	EC (2010)
Ethiopia	International	ARSO	2022	Maize	5	10	ARSO (2022)
Ethiopia	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Finland	International	EU	2002	Maize	2	4	EC (2010)
Finland	International	EU	2022	Maize	2	4	EC (2010)
Finland	International	EU	2002	Peanut	2	4	EC (2010)
Finland	International	EU	2022	Peanut	2	4	EC (2010)
France	International	EU	2002	Maize	2	4	EC (2010)
France	International	EU	2022	Maize	2	4	EC (2010)
France	International	EU	2002	Peanut	2	4	EC (2010)
France	International	EU	2022	Peanut	2	4	EC (2010)
Gabon	International	ARSO	2022	Maize	5	10	ARSO (2022)
Gabon	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Germany	International	EU	2002	Maize	2	4	EC (2010)
Germany	International	EU	2022	Maize	2	4	EC (2010)
Germany	International	EU	2002	Peanut	2	4	EC (2010)
Germany	International	EU	2022	Peanut	2	4	EC (2010)
Ghana	International	ARSO	2022	Maize	5	10	ARSO (2022)
Ghana	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Greece	International	EU	2002	Maize	2	4	EC (2010)
Greece	International	EU	2022	Maize	2	4	EC (2010)
Greece	International	EU	2002	Peanut	2	4	EC (2010)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Greece	International	EU	2022	Peanut	2	4	EC (2010)
Guatemala	National	-	2002	Maize	NA	20	Van Egmond and Jonker (2004)
Guatemala	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Guinea	International	ARSO	2022	Maize	5	10	ARSO (2022)
Guinea	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Guinea-Bissau	International	ARSO	2022	Maize	5	10	ARSO (2022)
Guinea-Bissau	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Honduras	National	-	2002	Maize	1	1	Van Egmond and Jonker (2004)
Honduras	National	-	2002	Peanut	NA	1	Van Egmond and Jonker (2004)
Hong Kong	National	-	2002	Maize	15	15	Van Egmond and Jonker (2004)
Hong Kong	National	-	2002	Peanut	20	20	Van Egmond and Jonker (2004)
Hungary	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Hungary	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Hungary	International	EU	2022	Maize	2	4	EC (2010)
Hungary	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Hungary	International	EU	2022	Peanut	2	4	EC (2010)
India	National	-	2002	Maize	NA	30	Van Egmond and Jonker (2004)
India	National	-	2022	Maize	10	15	FSSAI (2011)
India	National	-	2002	Peanut	NA	30	Van Egmond and Jonker (2004)
India	National	-	2022	Peanut	10	15	FSSAI (2011)
Indonesia	National	-	2022	Maize	15	20	MOA (2018)
Indonesia	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Indonesia	National	-	2022	Peanut	15	20	MOA (2018)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Iran	National	-	2002	Maize	5	30	Van Egmond and Jonker (2004)
Iran	National	-	2022	Maize	5	30	ISIRI (2002)
Iran	National	-	2002	Peanut	5	15	Van Egmond and Jonker (2004)
Iran	National	-	2022	Peanut	5	15	ISIRI (2002)
Ireland	International	EU	2002	Maize	2	4	EC (2010)
Ireland	International	EU	2022	Maize	2	4	EC (2010)
Ireland	International	EU	2002	Peanut	2	4	EC (2010)
Ireland	International	EU	2022	Peanut	2	4	EC (2010)
Israel	National	-	2002	Maize	5	15	Van Egmond and Jonker (2004)
Israel	National	-	2002	Peanut	5	15	Van Egmond and Jonker (2004)
Italy	International	EU	2002	Maize	2	4	EC (2010)
Italy	International	EU	2022	Maize	2	4	EC (2010)
Italy	International	EU	2002	Peanut	2	4	EC (2010)
Italy	International	EU	2022	Peanut	2	4	EC (2010)
Ivory Coast	International	ARSO	2022	Maize	5	10	ARSO (2022)
Ivory Coast	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Jamaica	National	-	2002	Maize	NA	20	Van Egmond and Jonker (2004)
Jamaica	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Japan	National	-	2002	Maize	NA	10	Van Egmond and Jonker (2004)
Japan	National	-	2022	Maize	10	NA	FAMIC (2015)
Japan	National	-	2002	Peanut	NA	10	Van Egmond and Jonker (2004)
Japan	National	-	2022	Peanut	10	NA	FAMIC (2015)
Jordan	National	-	2002	Maize	15	30	Van Egmond and Jonker (2004)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Jordan	National	-	2002	Peanut	15	30	Van Egmond and Jonker (2004)
Kazakhstan	International	EACU	2022	Maize	5	NA	EACU (2011)
Kazakhstan	International	EACU	2022	Peanut	5	NA	EACU (2011)
Kenya	International	ARSO	2022	Maize	5	10	ARSO (2022)
Kenya	International	EAC	2022	Maize	5	10	EAC (2018)
Kenya	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Kenya	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Kenya	International	EAC	2022	Peanut	5	10	EAC (2018)
Kuweit	International	-	2022	Maize	NA	4	Van Egmond and Jonker (2004)
Kuweit	International	-	2022	Peanut	NA	4	Van Egmond and Jonker (2004)
Kyrgyzstan	International	EACU	2022	Maize	5	NA	EACU (2011)
Kyrgyzstan	International	EACU	2022	Peanut	5	NA	EACU (2011)
Latvia	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Latvia	National	-	2002	Maize	5	NA	Van Egmond and Jonker (2004)
Latvia	International	EU	2022	Maize	2	4	EC (2010)
Latvia	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Latvia	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Latvia	International	EU	2022	Peanut	2	4	EC (2010)
Liberia	International	ARSO	2022	Maize	5	10	ARSO (2022)
Liberia	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Libya	International	ARSO	2022	Maize	5	10	ARSO (2022)
Libya	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Lithuania	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Lithuania	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Lithuania	International	EU	2022	Maize	2	4	EC (2010)
Lithuania	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Lithuania	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Lithuania	International	EU	2022	Peanut	2	4	EC (2010)
Luxembourg	International	EU	2002	Maize	2	4	EC (2010)
Luxembourg	International	EU	2022	Maize	2	4	EC (2010)
Luxembourg	International	EU	2002	Peanut	2	4	EC (2010)
Luxembourg	International	EU	2022	Peanut	2	4	EC (2010)
Madagascar	International	ARSO	2022	Maize	5	10	ARSO (2022)
Madagascar	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Malawi	International	ARSO	2022	Maize	5	10	ARSO (2022)
Malawi	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Malawi	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Malawi	National	-	2022	Peanut	NA	3	Chilaka et al. (2022)
Malaysia	National	-	2002	Maize	NA	35	Van Egmond and Jonker (2004)
Malaysia	National	-	2022	Maize	NA	5	MOH (2014)
Malaysia	National	-	2002	Peanut	NA	35	Van Egmond and Jonker (2004)
Malaysia	National	-	2022	Peanut	NA	10	MOH (2014)
Malta	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Malta	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Malta	International	EU	2022	Maize	2	4	EC (2010)
Malta	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Malta	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Malta	International	EU	2022	Peanut	2	4	EC (2010)
Mauritius	National	-	2002	Maize	5	10	Van Egmond and Jonker (2004)
Mauritius	International	ARSO	2022	Maize	5	10	ARSO (2022)
Mauritius	National	-	2002	Peanut	5	15	Van Egmond and Jonker (2004)
Mauritius	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Mexico	National	-	2002	Maize	NA	20	Van Egmond and Jonker (2004)
Mexico	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Moldova	National	-	2002	Maize	5	NA	Van Egmond and Jonker (2004)
Moldova	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Morocco	National	-	2002	Maize	5	NA	Van Egmond and Jonker (2004)
Morocco	International	ARSO	2022	Maize	5	10	ARSO (2022)
Morocco	National	-	2022	Maize	2	4	Chilaka et al. (2022)
Morocco	National	-	2002	Peanut	1	NA	Van Egmond and Jonker (2004)
Morocco	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Morocco	National	-	2022	Peanut	2	4	Chilaka et al. (2022)
Mozambique	National	-	2002	Peanut	NA	10	Van Egmond and Jonker (2004)
Namibia	International	ARSO	2022	Maize	5	10	ARSO (2022)
Namibia	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Nepal	National	-	2002	Maize	20	NA	Van Egmond and Jonker (2004)
Netherlands	International	EU	2002	Maize	2	4	EC (2010)
Netherlands	International	EU	2022	Maize	2	4	EC (2010)
Netherlands	International	EU	2002	Peanut	2	4	EC (2010)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Netherlands	International	EU	2022	Peanut	2	4	EC (2010)
New Zealand	International	AU&NZ	2002	Peanut	NA	15	FSANZ (2022)
Niger	International	ARSO	2022	Maize	5	10	ARSO (2022)
Niger	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Nigeria	National	-	2002	Maize	20	NA	Van Egmond and Jonker (2004)
Nigeria	International	ARSO	2022	Maize	5	10	ARSO (2022)
Nigeria	National	-	2002	Peanut	20	NA	Van Egmond and Jonker (2004)
Nigeria	International	ARSO	2022	Peanut	5	10	ARSO (2022)
North Macedonia	National	-	2022	Maize	2	4	AHV (2013)
North Macedonia	National	-	2022	Peanut	2	4	AHV (2013)
Norway	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Norway	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Oman	National	-	2002	Maize	10	NA	Van Egmond and Jonker (2004)
Oman	International	-	2022	Maize	NA	4	Van Egmond and Jonker (2004)
Oman	National	-	2002	Peanut	10	NA	Van Egmond and Jonker (2004)
Oman	International	-	2022	Peanut	NA	4	Van Egmond and Jonker (2004)
Paraguay	International	Mercosur	2002	Maize	NA	20	Mercosur (2002)
Paraguay	International	Mercosur	2002	Peanut	NA	20	Mercosur (2002)
Peru	National	-	2002	Peanut	NA	15	Van Egmond and Jonker (2004)
Philippines	National	-	2022	Maize	NA	20	BAFPS (2015)
Philippines	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Philippines	National	-	2022	Peanut	NA	15	BAFPS (2015)
Poland	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Poland	International	EU	2022	Maize	2	4	EC (2010)
Poland	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Poland	International	EU	2022	Peanut	2	4	EC (2010)
Portugal	International	EU	2002	Maize	2	4	EC (2010)
Portugal	International	EU	2022	Maize	2	4	EC (2010)
Portugal	International	EU	2002	Peanut	2	4	EC (2010)
Portugal	International	EU	2022	Peanut	2	4	EC (2010)
Qatar	International	-	2022	Maize	NA	4	Van Egmond and Jonker (2004)
Qatar	International	-	2022	Peanut	NA	4	Van Egmond and Jonker (2004)
Republic of Congo	International	ARSO	2022	Maize	5	10	ARSO (2022)
Republic of Congo	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Romania	National	-	2002	Maize	5	NA	Van Egmond and Jonker (2004)
Romania	International	EU	2022	Maize	2	4	EC (2010)
Romania	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Romania	International	EU	2022	Peanut	2	4	EC (2010)
Russia	National	-	2002	Maize	5	NA	Van Egmond and Jonker (2004)
Russia	International	EACU	2022	Maize	5	NA	EACU (2011)
Russia	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Russia	International	EACU	2022	Peanut	5	NA	EACU (2011)
Rwanda	International	ARSO	2022	Maize	5	10	ARSO (2022)
Rwanda	International	EAC	2022	Maize	5	10	EAC (2018)
Rwanda	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Rwanda	International	EAC	2022	Peanut	5	10	EAC (2018)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Salvador	National	-	2002	Maize	NA	20	Van Egmond and Jonker (2004)
Salvador	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Saudi Arabia	International	-	2022	Maize	NA	4	Van Egmond and Jonker (2004)
Saudi Arabia	International	-	2022	Peanut	NA	4	Van Egmond and Jonker (2004)
Senegal	International	ARSO	2022	Maize	5	10	ARSO (2022)
Senegal	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Serbia	National	-	2002	Maize	5	NA	Van Egmond and Jonker (2004)
Serbia	National	-	2022	Maize	2	4	RS (2019)
Serbia	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Serbia	National	-	2022	Peanut	2	4	RS (2019)
Seychelles	International	ARSO	2022	Maize	5	10	ARSO (2022)
Seychelles	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Sierra Leone	International	ARSO	2022	Maize	5	10	ARSO (2022)
Sierra Leone	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Singapore	National	-	2002	Maize	NA	5	Van Egmond and Jonker (2004)
Singapore	National	-	2022	Maize	5	5	SFA (2019)
Singapore	National	-	2002	Peanut	NA	5	Van Egmond and Jonker (2004)
Singapore	National	-	2022	Peanut	5	5	SFA (2019)
Slovakia	National	-	2002	Maize	20	80	Van Egmond and Jonker (2004)
Slovakia	International	EU	2022	Maize	2	4	EC (2010)
Slovakia	National	-	2002	Peanut	10	80	Van Egmond and Jonker (2004)
Slovakia	International	EU	2022	Peanut	2	4	EC (2010)
Slovenia	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Slovenia	International	EU	2022	Maize	2	4	EC (2010)
Slovenia	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Slovenia	International	EU	2022	Peanut	2	4	EC (2010)
Somalia	International	ARSO	2022	Maize	5	10	ARSO (2022)
Somalia	International	ARSO	2022	Peanut	5	10	ARSO (2022)
South Africa	National	-	2002	Maize	5	10	Van Egmond and Jonker (2004)
South Africa	International	ARSO	2022	Maize	5	10	ARSO (2022)
South Africa	National	-	2002	Peanut	5	10	Van Egmond and Jonker (2004)
South Africa	International	ARSO	2022	Peanut	5	10	ARSO (2022)
South Korea	National	-	2002	Maize	10	NA	Van Egmond and Jonker (2004)
South Korea	National	-	2022	Maize	10	15	MFDS (2019)
South Korea	National	-	2002	Peanut	10	NA	Van Egmond and Jonker (2004)
South Korea	National	-	2022	Peanut	10	15	MFDS (2019)
South Sudan	International	ARSO	2022	Maize	5	10	ARSO (2022)
South Sudan	International	EAC	2022	Maize	5	10	EAC (2018)
South Sudan	International	ARSO	2022	Peanut	5	10	ARSO (2022)
South Sudan	International	EAC	2022	Peanut	5	10	EAC (2018)
Spain	International	EU	2002	Maize	2	4	EC (2010)
Spain	International	EU	2022	Maize	2	4	EC (2010)
Spain	International	EU	2002	Peanut	2	4	EC (2010)
Spain	International	EU	2022	Peanut	2	4	EC (2010)
Sri Lanka	National	-	2002	Maize	NA	30	Van Egmond and Jonker (2004)
Sri Lanka	National	-	2002	Peanut	NA	30	Van Egmond and Jonker (2004)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Sudan	International	ARSO	2022	Maize	5	10	ARSO (2022)
Sudan	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Suriname	National	-	2002	Maize	NA	30	Van Egmond and Jonker (2004)
Suriname	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Swaziland	International	ARSO	2022	Maize	5	10	ARSO (2022)
Swaziland	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Sweden	International	EU	2002	Maize	2	4	EC (2010)
Sweden	International	EU	2022	Maize	2	4	EC (2010)
Sweden	International	EU	2002	Peanut	2	4	EC (2010)
Sweden	International	EU	2022	Peanut	2	4	EC (2010)
Switzerland	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Switzerland	National	-	2022	Maize	2	4	FDHA (2016)
Switzerland	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Switzerland	National	-	2022	Peanut	2	4	FDHA (2016)
Syria	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Taiwan	National	-	2002	Maize	NA	15	Van Egmond and Jonker (2004)
Taiwan	National	-	2022	Maize	5	10	TFDA (2019)
Taiwan	National	-	2002	Peanut	NA	15	Van Egmond and Jonker (2004)
Taiwan	National	-	2022	Peanut	2	4	TFDA (2019)
Tanzania	National	-	2002	Maize	5	10	Van Egmond and Jonker (2004)
Tanzania	International	ARSO	2022	Maize	5	10	ARSO (2022)
Tanzania	International	EAC	2022	Maize	5	10	EAC (2018)
Tanzania	International	ARSO	2022	Peanut	5	10	ARSO (2022)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Tanzania	International	EAC	2022	Peanut	5	10	EAC (2018)
Thailand	National	-	2002	Maize	NA	20	Van Egmond and Jonker (2004)
Thailand	National	-	2022	Maize	NA	20	ACFS (2009)
Thailand	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Thailand	National	-	2022	Peanut	NA	20	ACFS (2014)
Togo	International	ARSO	2022	Maize	5	10	ARSO (2022)
Togo	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Tunisia	National	-	2002	Maize	2	NA	Van Egmond and Jonker (2004)
Tunisia	International	ARSO	2022	Maize	5	10	ARSO (2022)
Tunisia	National	-	2002	Peanut	2	NA	Van Egmond and Jonker (2004)
Tunisia	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Turkey	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Turkey	National	-	2002	Maize	2	4	Van Egmond and Jonker (2004)
Turkey	National	-	2022	Maize	2	4	GKGM (2011)
Turkey	National	-	2002	Peanut	2	4	Van Egmond and Jonker (2004)
Turkey	National	-	2002	Peanut	5	10	Van Egmond and Jonker (2004)
Turkey	National	-	2022	Peanut	5	10	GKGM (2011)
Uganda	International	ARSO	2022	Maize	5	10	ARSO (2022)
Uganda	International	EAC	2022	Maize	5	10	EAC (2018)
Uganda	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Uganda	International	EAC	2022	Peanut	5	10	EAC (2018)
UK	International	EU	2002	Maize	2	4	EC (2010)
UK	International	EU	2022	Maize	2	4	EC (2010)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
UK	International	EU	2002	Peanut	2	4	EC (2010)
UK	International	EU	2022	Peanut	2	4	EC (2010)
Ukraine	National	-	2002	Maize	5	NA	Van Egmond and Jonker (2004)
Ukraine	National	-	2022	Maize	2	4	MOZ (2013)
Ukraine	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Ukraine	National	-	2022	Peanut	2	4	MOZ (2013)
United Arab Emirates	International	-	2022	Maize	NA	4	Van Egmond and Jonker (2004)
United Arab Emirates	International	-	2022	Peanut	NA	4	Van Egmond and Jonker (2004)
Uruguay	International	Mercosur	2002	Maize	NA	20	Mercosur (2002)
Uruguay	International	Mercosur	2002	Peanut	NA	20	Mercosur (2002)
USA	National	-	2002	Maize	NA	20	Van Egmond and Jonker (2004)
USA	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Venezuela	National	-	2002	Maize	NA	20	Van Egmond and Jonker (2004)
Venezuela	International	Mercosur	2002	Maize	NA	20	Mercosur (2002)
Venezuela	National	-	2002	Peanut	NA	20	Van Egmond and Jonker (2004)
Venezuela	International	Mercosur	2002	Peanut	NA	20	Mercosur (2002)
Vietnam	National	-	2002	Maize	NA	10	Van Egmond and Jonker (2004)
Vietnam	National	-	2022	Maize	2	4	MOH (2011)
Vietnam	National	-	2002	Peanut	NA	10	Van Egmond and Jonker (2004)
Vietnam	National	-	2022	Peanut	2	4	MOH (2011)
Zambia	International	ARSO	2022	Maize	5	10	ARSO (2022)
Zambia	International	ARSO	2022	Peanut	5	10	ARSO (2022)
Zimbabwe	National	-	2002	Maize	5	NA	Van Egmond and Jonker (2004)

Table 8.2 continued from previous page

Country	Hierarchy	Economic union	Time stamp	Food	AFB1	sumAFs	Reference
Zimbabwe	International	ARSO	2022	Maize	5	10	ARSO (2022)
Zimbabwe	National	-	2002	Peanut	5	NA	Van Egmond and Jonker (2004)
Zimbabwe	International	ARSO	2022	Peanut	5	10	ARSO (2022)

[a] Aflatoxin regulation limits are expressed in $\mu g\ kg^{-1}$.

ARSO = African Organisation for Standardisation; AU&NZ = Free trade zone of Australia and New Zealand; EAC = East African Community; EACU = Eurasian Customs Union; EU = European Union; GSO = Gulf Cooperation Council Standardization Organization; Mercosur = Southern Common Market; NA = Not available.

Physical-Chemical Property Estimation for Aflatoxins

Only a limited number of experimental studies have been conducted on the physico-chemical properties of AFs, necessitating the use of estimation software applications. The property estimation softwares OCHEM, EPISuite, ACD/Labs and OPERA were used to predict the boiling point (T_b), melting point (T_m), vapor pressure ($Log(P_v)$), water solubility ($Log(c_{max,w})$), Henry coefficient ($Log(K_H)$), octanol-air partitioning coefficient ($Log(K_{OA})$), octanol-water partitioning coefficient ($Log(K_{OW})$), soil absorption coefficient ($Log(K_{OC})$) of the four primary AFs (AFB1, AFB2, AFG1, and AFG2) as well as two major metabolites (AFB2a and AFM1). These estimations were either taken from freely available online databases such as OCHEM, CompTox and ChemSpider or manually calculated using the EPI Suite program (version 4.11). The estimations derived from the individual models are presented in table 8.3. However, using these models resulted in a high variability in the predicted values from the different calculators. In this regard, Tebes-Stevens et al. (2018) demonstrated for different chemicals that no individual estimation model outperforms the others, because the performance of the calculators is based on chemical class and the property value. However, the authors found that the geometric mean and the median of the calculated values from these multiple calculators that use different estimation algorithms are recommended as more reliable estimates of the property value than the value from any single calculator. For that reason, the median values were used to derive the entry pathways and fate of AFs in soil as described in chapter 1.2 and presented in table 1.1.

Table 8.3: Estimations of physicochemical properties and partition coefficients for the four primary aflatoxins (AFB1, AFB2, AFG1, AFG2) and two key metabolites (AFB2a, AFM1). Estimations are derived from models within the EPI Suite software and data from online databases including OCHEM, CompTox, and Chemspider.

Substance	Property	Source	Model	Value
AFB1	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALogPS 3.0)	2.2
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALogPS 3.0)	-3.8
	T_m	OCHEM	OCHEM Melting Point (Melting Point prediction (best Estate))	240
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (Water solubility model based on logP and Melting Point)	-3.9
	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALOGPS 2.1 logP)	1.7
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALOGPS 2.1 logS)	-3.1
	$Log(K_H)$	CompTox	OPERA	-5.7
	T_b	CompTox	OPERA	404
	T_b	CompTox	ACD/Labs	528
	T_m	CompTox	OPERA	207
	$Log(P_v)$	CompTox	ACD/Labs	-10.5
	$Log(P_v)$	CompTox	OPERA	-8.9
	$Log(c_{max,w})$	CompTox	OPERA	-3.0
	$Log(c_{max,w})$	CompTox	ACD/Labs	0.8
	$Log(K_{OW})$	CompTox	OPERA	0.4
	$Log(K_{OW})$	CompTox	ACD/Labs	0.5
	$Log(K_{OC})$	CompTox	OPERA	4.7
	$Log(K_{OC})$	Chemspider	ACD/Labs	2.0

Table 8.3 continued from previous page

Substance	Property	Source	Model	Value
	$Log(K_{OW})$	EPISuite	EPISuite KOWWIN v1.69	1.2
	T_b	EPISuite	EPISuite MPBPWIN v1.43	474
	T_m	EPISuite	EPISuite MPBPWIN v1.43	200
	$Log(P_v)$	EPISuite	EPISuite MPBPWIN v1.43	-8.8
	$Log(c_{max,w})$	EPISuite	EPISuite WSKOW v1.42	-2.5
	$Log(c_{max,w})$	EPISuite	EPISuite from Fragments (v1.01 est)	-4.7
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 Bond method	-12.9
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 [VP/WSol estimate using EPI values]	-9.1
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, MCI method	1.8
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, Kow method	1.8
AFB2	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALogPS 3.0)	2
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALogPS 3.0)	-3.9
	T_m	OCHEM	OCHEM Melting Point (Melting Point prediction (best Estate))	230
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (Water solubility model based on logP and Melting Point)	-3.7
	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALOGPS 2.1 logP)	1.6
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALOGPS 2.1 logS)	-2.9
	$Log(K_H)$	CompTox	OPERA	-5.3
	T_b	CompTox	OPERA	381
	T_b	CompTox	TEST	455
	T_b	CompTox	ACD/Labs	521
	T_m	CompTox	OPERA	287

Table 8.3 continued from previous page

Substance	Property	Source	Model	Value
	T_m	CompTox	TEST	207
	$Log(P_v)$	CompTox	ACD/Labs	-10.2
	$Log(P_v)$	CompTox	TEST	-9.6
	$Log(P_v)$	CompTox	OPERA	-8.9
	$Log(c_{max,w})$	CompTox	TEST	-3.2
	$Log(c_{max,w})$	CompTox	OPERA	-2.8
	$Log(c_{max,w})$	CompTox	ACD/Labs	0.8
	$Log(K_{OW})$	CompTox	OPERA	0.5
	$Log(K_{OW})$	CompTox	ACD/Labs	0.4
	$Log(K_{OC})$	CompTox	OPERA	4.7
	$Log(K_{OC})$	Chemspider	ACD/Labs	1.9
	$Log(K_{OW})$	EPISuite	EPISuite KOWWIN v1.69	1.5
	T_b	EPISuite	EPISuite MPBPWIN v1.43	473
	T_m	EPISuite	EPISuite MPBPWIN v1.43	200
	$Log(P_v)$	EPISuite	EPISuite MPBPWIN v1.43	-9.8
	$Log(c_{max,w})$	EPISuite	EPISuite WSKOW v1.42	-2.7
	$Log(c_{max,w})$	EPISuite	EPISuite from Fragments (v1.01 est)	-4.6
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 Bond method	-14.5
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 [VP/WSol estimate using EPI values]	-12.9
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, MCI method	1.8

Table 8.3 continued from previous page

Substance	Property	Source	Model	Value
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, Kow method	1.9
AFG1	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALogPS 3.0)	2.2
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALogPS 3.0)	-3.5
	T_m	OCHEM	OCHEM Melting Point (Melting Point prediction (best Estate))	230
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (Water solubility model based on logP and Melting Point)	-3.8
	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALOGPS 2.1 logP)	1.8
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALOGPS 2.1 logS)	-2.9
	$Log(K_H)$	CompTox	OPERA	-8.2
	T_b	CompTox	OPERA	403
	T_b	CompTox	TEST	497
	T_b	CompTox	ACD/Labs	612
	T_m	CompTox	OPERA	245
	T_m	CompTox	TEST	228
	$Log(P_v)$	CompTox	ACD/Labs	-14.2
	$Log(P_v)$	CompTox	OPERA	-10.0
	$Log(c_{max,w})$	CompTox	TEST	-3.1
	$Log(c_{max,w})$	CompTox	OPERA	-6.0
	$Log(c_{max,w})$	CompTox	ACD/Labs	0.8
	$Log(K_{OW})$	CompTox	OPERA	1.8
	$Log(K_{OW})$	CompTox	ACD/Labs	-0.2
	$Log(K_{OC})$	CompTox	OPERA	5.3

Table 8.3 continued from previous page

Substance	Property	Source	Model	Value
	$Log(K_{OC})$	Chemspider	ACD/Labs	1.8
	$Log(K_{OW})$	EPISuite	EPISuite KOWWIN v1.69	0.5
	T_b	EPISuite	EPISuite MPBPWIN v1.43	511
	T_m	EPISuite	EPISuite MPBPWIN v1.43	218
	$Log(P_v)$	EPISuite	EPISuite MPBPWIN v1.43	-10.2
	$Log(c_{max,w})$	EPISuite	EPISuite WSKOW v1.42	-2.0
	$Log(c_{max,w})$	EPISuite	EPISuite from Fragments (v1.01 est)	-4.2
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 Bond method	-12.3
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 [VP/WSol estimate using EPI values]	-14.1
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, MCI method	1.9
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, Kow method	1.1
AFG2	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALogPS 3.0)	1.6
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALogPS 3.0)	-3.5
	T_m	OCHEM	OCHEM Melting Point (Melting Point prediction (best Estate))	220
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (Water solubility model based on logP and Melting Point)	-3.6
	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALOGPS 2.1 logP)	1.6
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALOGPS 2.1 logS)	-2.8
	$Log(K_H)$	CompTox	OPERA	-5.8
	T_b	CompTox	OPERA	401
	T_b	CompTox	ACD/Labs	603
	T_m	CompTox	OPERA	189

Table 8.3 continued from previous page

Substance	Property	Source	Model	Value
	$Log(P_v)$	CompTox	ACD/Labs	-13.7
	$Log(P_v)$	CompTox	OPERA	-9.9
	$Log(c_{max,w})$	CompTox	OPERA	-1.5
	$Log(c_{max,w})$	CompTox	ACD/Labs	0.8
	$Log(K_{OW})$	CompTox	OPERA	0.5
	$Log(K_{OW})$	CompTox	ACD/Labs	-0.3
	$Log(K_{OC})$	CompTox	OPERA	4.3
	$Log(K_{OC})$	Chemspider	ACD/Labs	1.7
	$Log(K_{OW})$	EPISuite	EPISuite KOWWIN v1.69	0.7
	T_b	EPISuite	EPISuite MPBPWIN v1.43	510
	T_m	EPISuite	EPISuite MPBPWIN v1.43	217
	$Log(P_v)$	EPISuite	EPISuite MPBPWIN v1.43	-9.9
	$Log(c_{max,w})$	EPISuite	EPISuite WSKOW v1.42	-2.2
	$Log(c_{max,w})$	EPISuite	EPISuite from Fragments (v1.01 est)	-4.1
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 Bond method	-14.0
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 [VP/WSol estimate using EPI values]	-13.5
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, MCI method	1.9
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, Kow method	1.2
AFB2a	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALogPS 3.0)	1.0
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALogPS 3.0)	-3.2
	T_m	OCHEM	OCHEM Melting Point (Melting Point prediction (best Estate))	240

Table 8.3 continued from previous page

Substance	Property	Source	Model	Value
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (Water solubility model based on logP and Melting Point)	-3.1
	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALOGPS 2.1 logP)	1.0
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALOGPS 2.1 logS)	-2.4
	$Log(K_H)$	CompTox	OPERA	-8.2
	T_b	CompTox	OPERA	389
	T_b	CompTox	TEST	485
	T_b	CompTox	ACD/Labs	575
	T_m	CompTox	OPERA	187
	T_m	CompTox	TEST	233
	$Log(P_v)$	CompTox	ACD/Labs	-13.3
	$Log(P_v)$	CompTox	TEST	-12.2
	$Log(P_v)$	CompTox	OPERA	-8.5
	$Log(c_{max,w})$	CompTox	TEST	-3.1
	$Log(c_{max,w})$	CompTox	OPERA	-1.0
	$Log(K_{OW})$	CompTox	OPERA	-215
	$Log(K_{OW})$	CompTox	ACD/Labs	-0.5
	$Log(K_{OC})$	CompTox	OPERA	4.3
	$Log(K_{OC})$	Chemspider	ACD/Labs	1.5
	$Log(K_{OW})$	EPISuite	EPISuite KOWWIN v1.69	-0.4
	T_b	EPISuite	EPISuite MPBPWIN v1.43	508.8
	T_m	EPISuite	EPISuite MPBPWIN v1.43	216.7

Table 8.3 continued from previous page

Substance	Property	Source	Model	Value
	$Log(P_v)$	EPISuite	EPISuite MPBPWIN v1.43	>0.1
	$Log(c_{max,w})$	EPISuite	EPISuite WSKOW v1.42	-0.7
	$Log(c_{max,w})$	EPISuite	EPISuite from Fragments (v1.01 est)	-2.8
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 Bond method	-19.0
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 [VP/WSol estimate using EPI values]	-17.2
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, MCI method	1
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, Kow method	0.4
AFM1	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALogPS 3.0)	1.5
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALogPS 3.0)	-3.5
	T_m	OCHEM	OCHEM Melting Point (Melting Point prediction (best Estate))	230
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (Water solubility model based on logP and Melting Point)	-3.3
	$Log(K_{OW})$	OCHEM	OCHEM logPow (ALOGPS 2.1 logP)	1.2
	$Log(c_{max,w})$	OCHEM	OCHEM Water solubility (ALOGPS 2.1 logS)	-2.5
	$Log(K_H)$	CompTox	OPERA	-8.7
	T_b	CompTox	OPERA	409
	T_b	CompTox	ACD/Labs	644
	T_m	CompTox	OPERA	170
	$Log(P_v)$	CompTox	ACD/Labs	-16.8
	$Log(P_v)$	CompTox	OPERA	-8.6
	$Log(c_{max,w})$	CompTox	OPERA	-1.2
	$Log(c_{max,w})$	CompTox	ACD/Labs	0.8

Table 8.3 continued from previous page

Substance	Property	Source	Model	Value
	$Log(K_{OW})$	CompTox	OPERA	-0.2
	$Log(K_{OW})$	CompTox	ACD/Labs	-0.3
	$Log(K_{OC})$	CompTox	OPERA	4.3
	$Log(K_{OC})$	Chemspider	ACD/Labs	1.5
	$Log(K_{OW})$	EPISuite	EPISuite KOWWIN v1.69	-0.3
	T_b	EPISuite	EPISuite MPBPWIN v1.43	502
	T_m	EPISuite	EPISuite MPBPWIN v1.43	214
	$Log(P_v)$	EPISuite	EPISuite MPBPWIN v1.43	-11.8
	$Log(c_{max,w})$	EPISuite	EPISuite WSKOW v1.42	-0.9
	$Log(c_{max,w})$	EPISuite	EPISuite from Fragments (v1.01 est)	-2.7
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 Bond method	-17.3
	$Log(K_H)$	EPISuite	EPISuite HENRYWIN v3.20 [VP/WSol estimate using EPI values]	-16.8
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, MCI method	1
	$Log(K_{OC})$	EPISuite	EPISuite PCKOCWIN v1.66, Kow method	0.5

T_b = Boiling point; T_m = Melting point; $Log(P_v)$ = Vapor pressure, logarithmic scale; $Log(c_{max,w})$ = Water solubility, logarithmic scale; $Log(K_{OA})$ = Octanol-Air-partitioning coefficient, logarithmic scale; $Log(K_{OW})$ = Octanol-Water-partitioning coefficient, logarithmic scale; $Log(K_H)$ = Henry coefficent, logarithmic scale; $Log(K_{OC})$ = Soil absorption coefficient, logarithmic scale.

8.2 Supporting Information on Chapter 2

Supporting Information

Validation of a simple and reliable method for the determination of aflatoxins in soil and food matrices

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Table SI-1. Validation parameters for the mycotoxins investigated in different soil and food matrices using LC-MS and HPLC-FLD. SSE = signal suppression/enhancement, w_i = weighting factor used for weighted calibration, R_{WLS}^2 = coefficient of determination of the weighted calibration, R_{OLS}^2 = coefficient of determination of the unweighted model, $RE_{sum,WLS}$ = sum of relative errors of the weighted calibration model, $RE_{sum,OLS}$ = sum of relative errors of the unweighted calibration model, ΔRE_{sum} = reduction of the sum of relative errors due to application of weighting factor, LOD = limit of detection, LOQ = limit of quantification.

Matrix	Analyte	LC-MS									HPLC-FLD								
		SSE (%)	w_i	R^2_{WLS}	R^2_{OLS}	$RE_{sum,WLS}$ (%)	$RE_{sum,OLS}$ (%)	ΔRE_{sum} (%)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	SSE (%)	w_i	R_{WLS}^2	R_{OLS}^2	$RE_{sum,WLS}$ (%)	$RE_{sum,OLS}$ (%)	ΔRE_{sum} (%)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
Refesol 01-A	AFB1	-7	$1/y^2$	0.99	0.997	65	193	-66	0.031	0.104	-1	$1/x^2$	0.967	>0.999	97	145	-33	0.022	0.075
Refesol 01-A	AFB2	-5	$1/y^2$	0.987	0.995	61	99	-38	0.028	0.094	0	$1/x^2$	0.997	0.998	42	390	-89	0.025	0.085
Refesol 01-A	AFG1	-18	$1/y^2$	0.983	0.997	94	517	-82	0.037	0.122	-10	$1/y^1$	0.992	0.994	123	213	-43	0.025	0.082
Refesol 01-A	AFG2	-14	$1/y^2$	0.974	0.995	102	424	-76	0.033	0.11	-12	$1/x^2$	0.985	0.992	103	636	-84	0.046	0.154
Refesol 02-A	AFB1	-7	$1/x^2$	0.989	0.998	85	105	-20	0.039	0.128	-8	$1/y^2$	0.985	0.997	109	591	-82	0.041	0.136
Refesol 02-A	AFB2	-3	$1/x^2$	0.987	0.996	82	194	-58	0.019	0.062	-2	$1/y^2$	0.994	0.999	64	327	-80	0.02	0.066
Refesol 02-A	AFG1	-13	$1/x^2$	0.986	0.996	96	117	-18	0.039	0.129	-14	$1/x^2$	0.991	0.997	78	316	-75	0.027	0.09
Refesol 02-A	AFG2	-12	$1/x^2$	0.986	0.995	89	197	-55	0.028	0.092	-3	$1/y^1$	0.998	0.998	67	306	-78	0.026	0.088
LUFA 2.4	AFB1	-11	$1/x^2$	0.987	0.997	84	360	-77	0.047	0.156	-7	$1/x^2$	0.984	0.979	102	1490	-93	0.06	0.199
LUFA 2.4	AFB2	-12	$1/y^2$	0.993	0.996	64	128	-50	0.038	0.128	-3	$1/y^2$	0.993	0.999	65	397	-84	0.017	0.057
LUFA 2.4	AFG1	-23	$1/y^2$	0.992	0.996	71	265	-73	0.047	0.157	-6	$1/x^2$	0.989	0.987	80	1217	-93	0.031	0.104
LUFA 2.4	AFG2	-20	$1/x^1$	0.998	0.998	67	223	-70	0.044	0.145	0	$1/y^2$	0.997	0.999	44	401	-89	0.02	0.067
LUFA 6S	AFB1	-19	$1/x^2$	0.984	>0.999	82	275	-70	0.046	0.153	-3	$1/x^2$	0.986	0.996	95	651	-85	0.026	0.087
LUFA 6S	AFB2	-23	$1/y^2$	0.984	>0.999	75	202	-63	0.04	0.133	2	$1/y^1$	0.998	>0.999	166	281	-41	0.035	0.117
LUFA 6S	AFG1	-25	$1/y^1$	0.999	>0.999	84	160	-48	0.033	0.109	-14	$1/y^1$	0.996	0.997	178	327	-45	0.04	0.135
LUFA 6S	AFG2	-22	$1/x^2$	0.988	>0.999	65	194	-66	0.04	0.132	-1	$1/y^2$	0.996	0.998	44	537	-92	0.032	0.106
Maize	AFB1	-54	$1/x^2$	0.987	0.992	88	110	-20	0.039	0.13	-7	$1/x^2$	0.972	0.99	121	940	-87	0.026	0.088
Maize	AFB2	-46	$1/x^2$	0.989	0.997	76	208	-64	0.062	0.206	-6	$1/y^2$	0.999	0.999	28	211	-87	0.019	0.065
Maize	AFG1	-49	$1/x^2$	0.991	0.998	78	171	-55	0.051	0.171	2	$1/x^2$	0.988	0.991	80	982	-92	0.047	0.155
Maize	AFG2	-47	$1/x^2$	0.984	0.998	82	214	-62	0.062	0.207	0	$1/x^{0.5}$	0.999	0.999	38	122	-69	0.011	0.036
Wheat	AFB1	-45	$1/x^{0.5}$	0.999	>0.999	130	131	-1	0.041	0.138	-13	$1/x^1$	0.998	0.998	93	343	-73	0.038	0.126
Wheat	AFB2	-41	$1/x^2$	0.994	0.999	52	58	-11	0.029	0.097	-7	$1/x^2$	0.999	>0.999	28	191	-85	0.016	0.052
Wheat	AFG1	-36	$1/x^2$	0.991	0.999	81	277	-71	0.039	0.13	-8	$1/x^2$	0.99	0.989	74	846	-91	0.021	0.068
Wheat	AFG2	-39	$1/x^1$	0.996	0.998	141	365	-61	0.042	0.141	-4	$1/x^2$	0.998	>0.999	30	130	-77	0.016	0.053
Millet	AFB1	-43	$1/x^2$	0.983	0.998	103	232	-56	0.021	0.071	5	$1/y^2$	0.982	0.994	102	757	-87	0.059	0.195
Millet	AFB2	-47	$1/x^2$	0.987	0.995	83	87	-4	0.019	0.063	5	$1/x^2$	0.999	0.999	25	235	-89	0.028	0.095
Millet	AFG1	-46	$1/x^2$	0.986	0.994	100	360	-72	0.043	0.145	0	$1/x^2$	0.996	0.994	49	672	-93	0.027	0.088

Millet	AFG2	-43	$1/x^2$	0.989	0.994	84	314	-73	0.047	0.158	3	$1/y^2$	0.989	0.999	75	137	-45	0.01	0.035
Peanut	AFB1	-40	$1/y^2$	0.98	0.991	104	159	-35	0.056	0.186	-6	$1/x^2$	0.975	0.989	131	1383	-91	0.038	0.127
Peanut	AFB2	-47	$1/x^2$	0.988	0.998	85	104	-18	0.058	0.194	-4	$1/x^2$	0.992	>0.999	54	148	-64	0.017	0.056
Peanut	AFG1	-50	$1/x^2$	0.986	0.997	90	165	-45	0.041	0.136	-6	$1/x^2$	0.993	0.993	68	787	-91	0.05	0.167
Peanut	AFG2	-43	$1/x^2$	0.986	0.995	90	187	-52	0.047	0.157	-4	$1/y^1$	1	>0.999	34	92	-63	0.022	0.074
Pistachio	AFB1	-43	$1/y^2$	0.985	0.998	35	240	-86	0.062	0.208	9	$1/x^2$	0.966	0.997	130	732	-82	0.037	0.124
Pistachio	AFB2	-48	$1/y^2$	0.994	0.999	32	214	-85	0.069	0.23	-1	$1/x^2$	0.996	0.998	53	388	-86	0.01	0.034
Pistachio	AFG1	-52	$1/y^2$	0.997	0.999	20	173	-88	0.068	0.226	4	$1/x^2$	0.953	0.999	144	632	-77	0.026	0.085
Pistachio	AFG2	-53	$1/y^2$	0.991	0.999	32	237	-86	0.063	0.209	5	$1/y^2$	0.996	0.998	58	534	-89	0.017	0.056

Table SI-2. Mean and relative standard deviation (in brackets) of recoveries for the mycotoxins investigated in different soil and food matrices at three fortification levels (N=10 each) and overall (N=30).

Matrix	Analyte	Recovery			
		0.5 µg kg^{-1}	5 µg kg^{-1}	20 µg kg^{-1}	Overall
Refesol 01-A	AFB1	64(10)	87(3)	87(3)	80(15)
Refesol 01-A	AFB2	71(8)	89(3)	91(3)	84(12)
Refesol 01-A	AFG1	74(10)	90(5)	97(5)	87(13)
Refesol 01-A	AFG2	77(9)	94(5)	96(4)	89(12)
Refesol 02-A	AFB1	76(11)	85(3)	87(2)	83(8)
Refesol 02-A	AFB2	80(5)	87(4)	85(2)	84(5)
Refesol 02-A	AFG1	76(11)	85(6)	87(4)	83(9)
Refesol 0-2A	AFG2	75(8)	84(5)	83(4)	81(8)
LUFA 2.4	AFB1	78(13)	81(11)	90(3)	83(11)
LUFA 2.4	AFB2	74(11)	74(12)	85(2)	78(11)
LUFA 2.4	AFG1	87(11)	88(12)	99(9)	92(12)
LUFA 2.4	AFG2	77(12)	77(11)	90(8)	81(12)
LUFA 6S	AFB1	81(12)	87(5)	84(7)	84(8)
LUFA 6S	AFB2	81(10)	88(4)	82(5)	84(8)
LUFA 6S	AFG1	82(8)	83(6)	81(5)	82(7)
LUFA 6S	AFG2	77(11)	84(4)	78(4)	80(7)
Maize	AFB1	92(9)	90(5)	93(4)	92(6)
Maize	AFB2	84(15)	83(8)	81(4)	83(10)
Maize	AFG1	88(12)	84(7)	87(7)	86(9)
Maize	AFG2	73(18)	82(5)	85(6)	80(12)
Wheat	AFB1	72(12)	89(7)	95(4)	85(14)
Wheat	AFB2	73(8)	81(5)	85(3)	79(8)
Wheat	AFG1	78(11)	82(5)	90(3)	83(9)
Wheat	AFG2	75(12)	88(3)	89(4)	84(10)
Millet	AFB1	83(5)	83(4)	82(2)	83(4)
Millet	AFB2	75(5)	85(4)	83(2)	81(7)
Millet	AFG1	85(11)	90(4)	88(2)	88(7)
Millet	AFG2	76(13)	89(6)	84(2)	83(10)
Peanut	AFB1	76(15)	88(7)	97(3)	87(13)
Peanut	AFB2	78(16)	88(9)	96(4)	88(13)
Peanut	AFG1	86(10)	83(8)	93(6)	87(9)
Peanut	AFG2	88(11)	88(9)	91(4)	89(8)
Pistachio	AFB1	92(14)	95(4)	96(3)	94(8)
Pistachio	AFB2	80(18)	96(7)	99(5)	92(14)
Pistachio	AFG1	79(18)	100(5)	101(3)	93(15)
Pistachio	AFG2	92(14)	101(5)	103(3)	99(10)

Table SI-3. Summary of statistic models: Effects of weighting (Weighting), intrument (LC-MS, HPLC-FLD), matrix type (soil, food), fortification level (low, medium, high) and their interactions on coefficient of determination (R_{adj}^2), sum of percentage relative error (RE_{sum}(%)), matrix effect (|SSE|), limit of detection and quantification (LOD, LOQ), relative spike recovery (Recovery) and relative standard deviation of spike recovery (RSD_r).

Parameter	Predictor	DF	Test statistics	Value	p
R^2_{adj}	Weighting	79	Paired T-Test	7.52	<0.001
RE_{sum}(%)	Weighting	79	Paired T-Test	-8.33	<0.001
|SSE|	Instrument	1	F-ANOVA	512.1	<0.001
|SSE|	Matrix type	1	F-ANOVA	165.6	<0.001
|SSE|	Instrument: Matrix type	1	F-ANOVA	174.9	<0.001
LOD	Instrument	1	F-ANOVA	24.07	<0.001
LOD	Matrix type	1	F-ANOVA	1.43	0.24
LOD	Instrument: Matrix type	1	F-ANOVA	6.58	0.01
LOQ	Instrument	1	F-ANOVA	24.19	<0.001
LOQ	Matrix type	1	F-ANOVA	1.49	0.23
LOQ	Instrument: Matrix type	1	F-ANOVA	7.01	0.01
Recovery	Matrix type	1	F-ANOVA	5.62	0.02
Recovery	Fortification level	2	F-ANOVA	21.36	<0.001
Recovery	Matrix type: Fortification level	2	F-ANOVA	0.44	0.44
Recovery	Clay content	44	T-Test	-0.67	0.51
Recovery	CEC	44	T-Test	-0.89	0.38
Recovery	C_{org}	44	T-Test	-0.69	0.49
RSD_r	Matrix type	1	F-ANOVA	1.31	0.25
RSD_r	Fortification level	2	F-ANOVA	91.49	<0.001
RSD_r	Matrix type: Fortification level	2	F-ANOVA	4.99	0.01

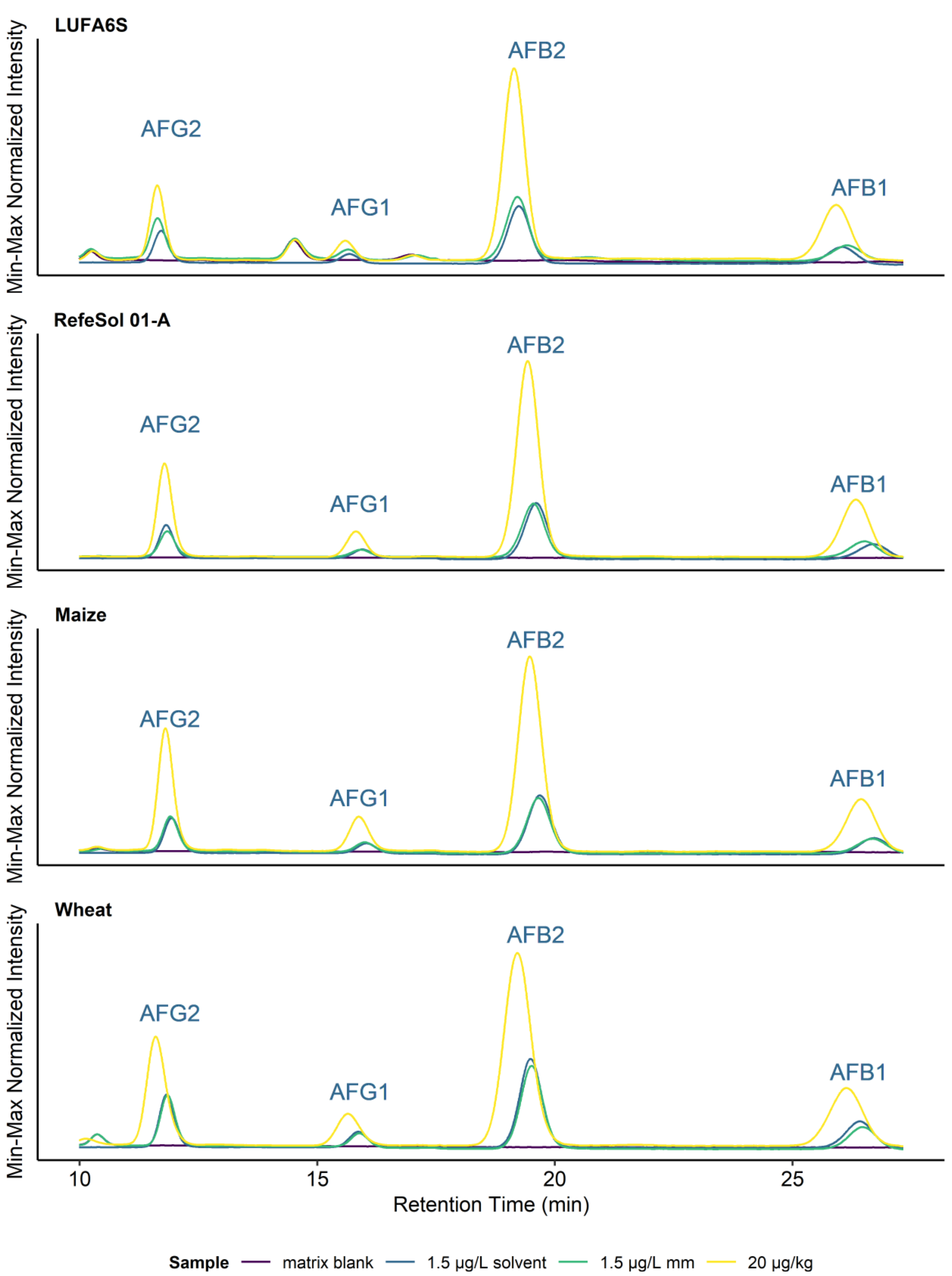

Figure SI-1. HPLC-FLD chromatograms obtained from injection of spiked samples (fortification level 20 µg kg^{-1}), sample blank and matrix standard solution (1.5 µg L^{-1}) of the soils Refesol 01-A and LUFA6 and food matrices maize and wheat (highlighted by different colors).

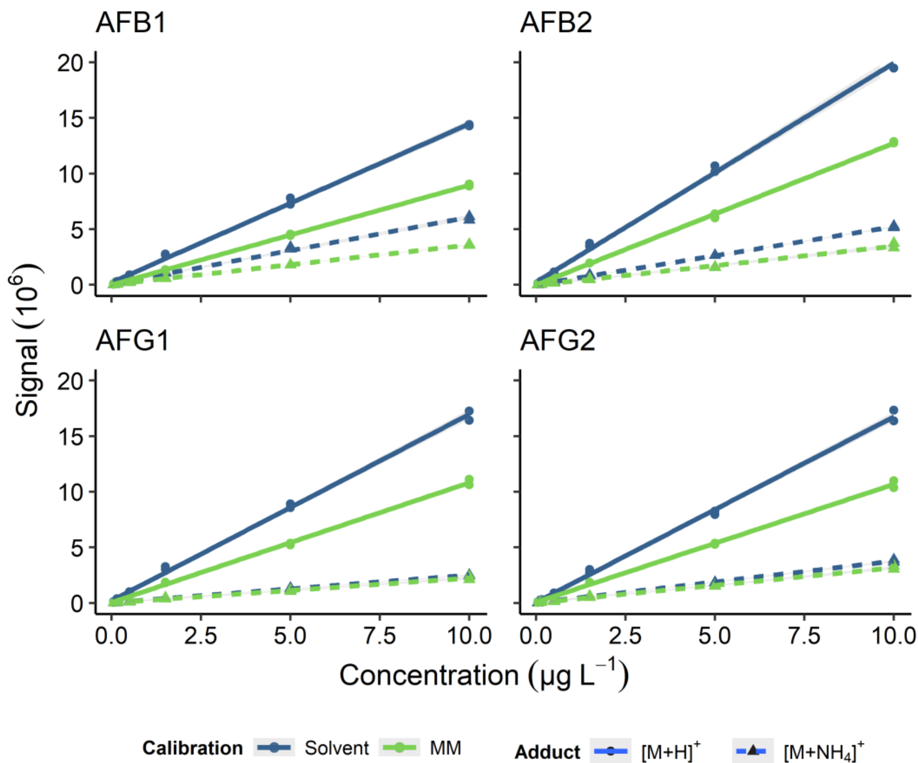

Figure SI-2. Solvent (green) and matrix-matched (blue) calibration for Wheat samples performed with the $[M+H]^+$ (solid) and $[M+NH4]^+$ (dashed) adducts

8.3 Supporting Information on Chapter 3

Standard Operating Procedure for Field Sampling of Soil for Mycotoxin Analysis

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By Julius Albert and Katherine Munoz

June 2020

Scope and Field of Application

The provided standard operating procedures are designed to assist researchers and practitioners in effectively sampling agricultural soil for the determination of mycotoxin field levels. Accurately assessing mycotoxin occurrence and distribution within a field requires the utilization of appropriate, reliable, and reproducible sampling techniques. Agricultural soils possess inherent heterogeneity, both across the field and down the soil profile, leading to highly variable mycotoxin levels even on small scales. This inherent variability emphasizes the need for careful sampling to ensure that the collected samples truly represent the occurrence and distribution in the field. When aiming to determine representative mean levels of mycotoxins on a field scale, multiple clusters should be selected in the field. From each cluster, numerous individual samples must be collected at different positions (between plants and inter-row) and depths (topsoil and subsoil) using a fine mesh of sampling points. These samples should be adequately homogenized to obtain pooled samples that accurately represent the respective positions and depths of each cluster. By following these instructions, researchers and practitioners can obtain reliable and representative data on mycotoxin levels in agricultural soils. This information contributes to the development of effective management strategies and supports decision-making in the agricultural sector.

Materials

The following materials are required for field sampling:

- Appropriate sample container e.g. plastic Bags (minimum 0.5 L)
- Large Spoon
- Auger
- Large wooden/plastic hammer
- Knife
- Permanent Marker

- Portable Balance
- Meterstick
- Buckets
- Deionized water

Preliminary Preparation

Before and after sampling, clean the sampling equipment with deionized water to reduce sample carryover and contamination. This includes the auger, buckets, spoon and knife. If necessary, and especially when changing to another field, it is advisable to draw a soil core once and then discard it. In this way, the equipment is pre-rinsed with the new field soil.

Soil Sampling Procedure

In a first step, a minimum of 5 clusters are assigned in the field and labeled with a unique identifier. Each cluster should consist of a minimum of 10 crop plants. A minimum of 5 individual samples are taken between the plants in the plant row, and an additional 5 sampling points are designated at the same height in the inter-row position (Figure 8.1).

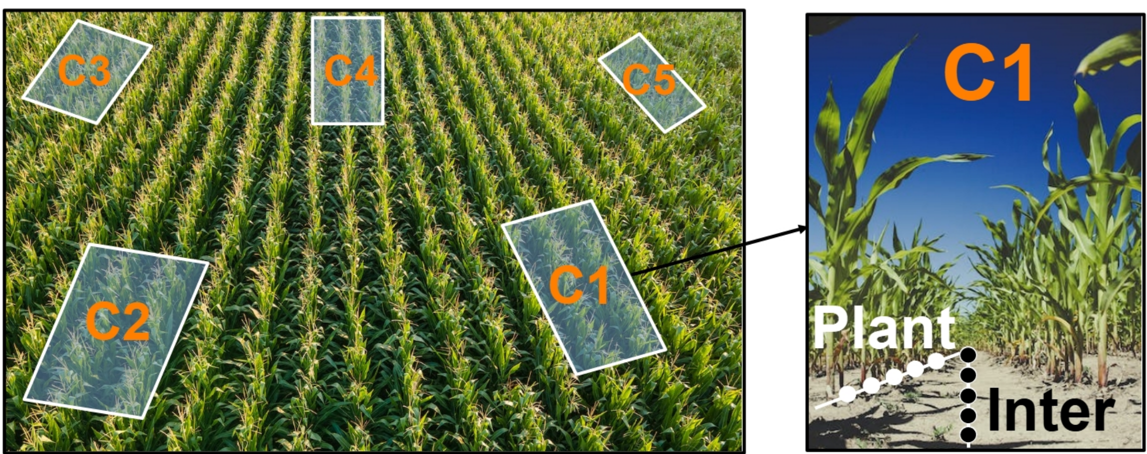

Figure 8.1: Scheme for field sampling of soil for mycotoxin analysis. A minimum of 5 clusters are assigned in the field. In each cluster, 5 individual sampling positions are designated between the plants in the planting row ("Plant") and 5 samples are designated in the inter-row ("Inter").

Four sampling buckets are provided to collect the individual samples for each combination of position (at-plant and inter-row) and depth (topsoil and subsoil). At each sampling point, the auger is drilled straight into the ground using the sampling hammer to obtain a soil core of approximately 35-40 cm. The soil core is then divided into three sections by making a cut with a knife: 0-15 cm, 15-30 cm, and >30 cm. The last fraction is discarded. The 0-15 cm section is collected in the respective topsoil sampling bucket for each position, while the 15-30 cm section is collected in the respective subsoil sampling bucket (Figure 8.2). The individual samples from each position and depth within a cluster are combined in their respective buckets and thoroughly mixed using a spoon. Large rocks and plant materials are to be removed. Approximately 300 g of the homogenized sample is then placed into the corresponding sample bag.

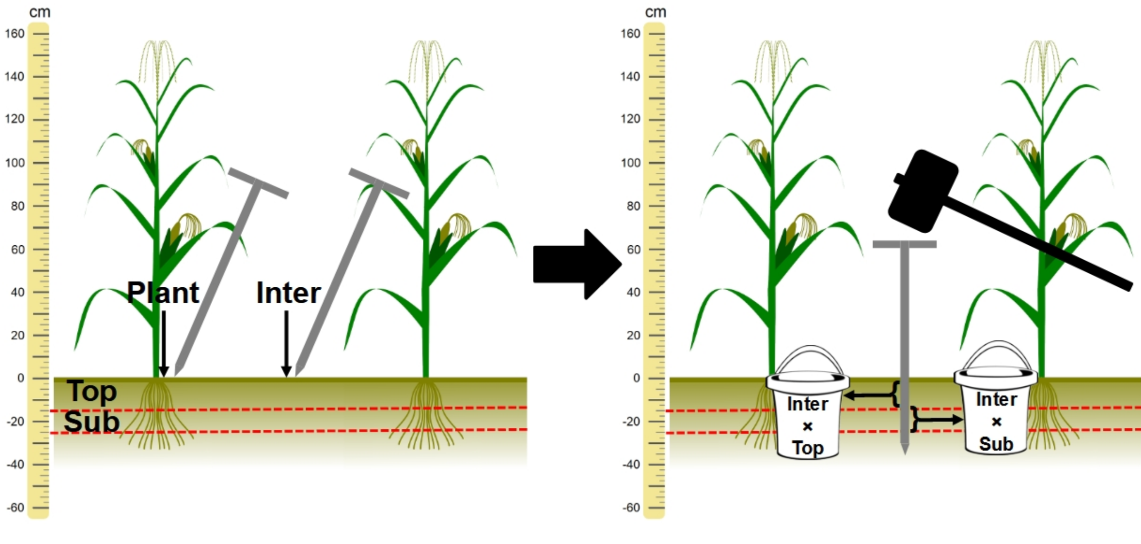

Figure 8.2: Soil sampling procedure. At each cluster, soil cores are taken at the "Plant" and "Inter" positions using a soil auger. The soil cores are then divided into "upper" (0-15 cm) and "lower" soil layers (15-30 cm). The individual samples for each position and depth are collected in buckets and homogenized by mixing with a spoon to obtain a homogenized composite sample.

Packaging, Storage and Transport

To ensure the preservation and integrity of samples during storage or transport, it is important to use appropriate, robust and non-reactive packaging materials, such as plastic or paper bags. This choice of packaging will help protect samples from moisture, pests, spills, and contamination. Since most mycotoxins are susceptible to

photolytic and microbial degradation, it is advisable to store samples in a cool, light-protected environment as soon as possible after collection. For short-term storage of a few weeks, a temperature of at least 4 °C is recommended, while for longer-term storage over months, a temperature of at least -20 °C should be maintained.

Recording

Each pooled sample should be labeled accordingly. The label should contain the following information:

- Field number
- Cluster number
- Position (At-Plant or Inter-Row)
- Layer (Topsoil or Subsoil)
- Date

In addition, the following information should be collected during each field sampling and attached to the respective field batch of samples on an information sheet:

- Name of the collecting institution and personnel
- Photographs of the field, close-ups of the ground and crop plants
- Weather conditions
- Date
- Start and finish time
- GPS coordinates
- Optional: additional field parameters may be recorded, such as treatments.

8.4 Supporting Information on Chapter 4

Kinetics of microbial and photochemical degradation of aflatoxin B1 in a sandy loam and clay soil

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ABSTRACT

In this supplementary information, you will find the raw data as well as additional information on our experiments, chromatography and statistical analyses that support our results.

SI-1 Raw data for kinetic modeling of AFB1 dissipation

Table 1. Calculated AFB1 and AFB2a concentrations in the sterile (PD+C) and nonsterile (MD) soils fortified with 0.5. 5, 50, 250 and 500 µg kg^{-1} AFB1. Values below the detection limit (defined as below the lowest calibration standard i.e. 0.05 µg L^{-1}) are shown as < LOD.

Soil	Fortification level (µg kg^{-1})	Day	Replicate	Degradation Type	AFB1 (µg kg^{-1})	AFB2a (µg kg^{-1})
R01A	0.5	0	1	MD	0.32	-
R01A	0.5	0	2	MD	0.36	-
R01A	0.5	0	3	MD	0.32	-
R01A	0.5	1	1	MD	0.30	-
R01A	0.5	1	2	MD	0.28	-
R01A	0.5	1	3	MD	0.27	-
R01A	0.5	3	1	MD	0.24	-
R01A	0.5	3	2	MD	0.24	-
R01A	0.5	3	3	MD	0.24	-
R01A	0.5	8	1	MD	0.20	-
R01A	0.5	8	2	MD	0.22	-
R01A	0.5	8	3	MD	0.27	-
R01A	0.5	15	1	MD	0.21	-
R01A	0.5	15	2	MD	0.22	-
R01A	0.5	15	3	MD	0.20	-
R01A	0.5	22	1	MD	0.12	-
R01A	0.5	22	2	MD	0.13	-
R01A	0.5	22	3	MD	0.13	-
R01A	0.5	28	1	MD	0.11	-
R01A	0.5	28	2	MD	0.12	-
R01A	0.5	28	3	MD	0.13	-
L6S	0.5	0	1	MD	0.27	-
L6S	0.5	0	2	MD	0.32	-
L6S	0.5	0	3	MD	0.36	-
L6S	0.5	1	1	MD	0.28	-
L6S	0.5	1	2	MD	0.31	-

L6S	0.5	1	3	MD	0.34	-
L6S	0.5	3	1	MD	0.24	-
L6S	0.5	3	2	MD	0.24	-
L6S	0.5	3	3	MD	0.27	-
L6S	0.5	8	1	MD	0.27	-
L6S	0.5	8	2	MD	0.30	-
L6S	0.5	8	3	MD	0.27	-
L6S	0.5	15	1	MD	0.24	-
L6S	0.5	15	2	MD	0.26	-
L6S	0.5	15	3	MD	0.23	-
L6S	0.5	22	1	MD	0.21	-
L6S	0.5	22	2	MD	0.22	-
L6S	0.5	22	3	MD	0.24	-
L6S	0.5	28	1	MD	0.22	-
L6S	0.5	28	2	MD	0.24	-
L6S	0.5	28	3	MD	0.20	-
R01A	5	0	1	MD	4.34	-
R01A	5	0	2	MD	4.50	-
R01A	5	0	3	MD	4.70	-
R01A	5	1	1	MD	3.90	-
R01A	5	1	2	MD	3.72	-
R01A	5	1	3	MD	3.98	-
R01A	5	3	1	MD	3.74	-
R01A	5	3	2	MD	3.85	-
R01A	5	3	3	MD	3.56	-
R01A	5	8	1	MD	3.16	-
R01A	5	8	2	MD	3.37	-
R01A	5	8	3	MD	3.35	-
R01A	5	15	1	MD	2.50	-
R01A	5	15	2	MD	2.44	-
R01A	5	15	3	MD	2.49	-
R01A	5	22	1	MD	1.53	-
R01A	5	22	2	MD	2.03	-
R01A	5	22	3	MD	2.08	-
R01A	5	28	1	MD	1.54	-
R01A	5	28	2	MD	1.55	-
R01A	5	28	3	MD	1.59	-
L6S	5	0	1	MD	3.30	-
L6S	5	0	2	MD	3.32	-
L6S	5	0	3	MD	3.13	-
L6S	5	1	1	MD	3.26	-
L6S	5	1	2	MD	2.85	-
L6S	5	1	3	MD	3.13	-
L6S	5	3	1	MD	2.78	-
L6S	5	3	2	MD	2.86	-
L6S	5	3	3	MD	2.69	-
L6S	5	8	1	MD	2.42	-
L6S	5	8	2	MD	2.68	-
L6S	5	8	3	MD	2.78	-
L6S	5	15	1	MD	2.32	-
L6S	5	15	2	MD	2.61	-
L6S	5	15	3	MD	2.44	-
L6S	5	22	1	MD	2.20	-
L6S	5	22	2	MD	2.39	-
L6S	5	22	3	MD	2.10	-

L6S	5	28	1	MD	2.27	-
L6S	5	28	2	MD	1.93	-
L6S	5	28	3	MD	2.17	-
R01A	50	0	1	MD	35.09	0.00
R01A	50	0	2	MD	35.05	0.00
R01A	50	0	3	MD	35.72	0.00
R01A	50	1	1	MD	34.29	0.51
R01A	50	1	2	MD	34.57	0.59
R01A	50	1	3	MD	31.37	0.46
R01A	50	3	1	MD	33.06	0.86
R01A	50	3	2	MD	33.49	0.82
R01A	50	3	3	MD	32.91	0.80
R01A	50	8	1	MD	24.95	7.24
R01A	50	8	2	MD	27.90	0.85
R01A	50	8	3	MD	26.19	0.77
R01A	50	15	1	MD	22.19	0.78
R01A	50	15	2	MD	20.91	4.63
R01A	50	15	3	MD	20.35	11.11
R01A	50	22	1	MD	16.90	4.29
R01A	50	22	2	MD	17.59	4.05
R01A	50	22	3	MD	15.06	3.30
R01A	50	28	1	MD	14.59	4.46
R01A	50	28	2	MD	15.44	4.54
R01A	50	28	3	MD	11.56	11.42
L6S	50	0	1	MD	33.10	0.00
L6S	50	0	2	MD	33.07	0.00
L6S	50	0	3	MD	32.78	0.00
L6S	50	1	1	MD	30.22	0.19
L6S	50	1	2	MD	30.93	0.22
L6S	50	1	3	MD	33.18	0.42
L6S	50	3	1	MD	29.97	0.27
L6S	50	3	2	MD	31.53	0.30
L6S	50	3	3	MD	28.69	0.32
L6S	50	8	1	MD	29.80	0.50
L6S	50	8	2	MD	24.77	0.33
L6S	50	8	3	MD	28.28	0.43
L6S	50	15	1	MD	22.82	0.60
L6S	50	15	2	MD	22.38	0.58
L6S	50	15	3	MD	23.88	3.54
L6S	50	22	1	MD	21.72	0.58
L6S	50	22	2	MD	25.23	0.62
L6S	50	22	3	MD	21.49	0.57
L6S	50	28	1	MD	19.51	0.69
L6S	50	28	2	MD	17.81	0.52
L6S	50	28	3	MD	19.69	0.57
R01A	250	0	1	MD	169.54	-
R01A	250	0	2	MD	158.86	-
R01A	250	0	3	MD	166.07	-
R01A	250	1	1	MD	159.52	-
R01A	250	1	2	MD	158.09	-
R01A	250	1	3	MD	171.29	-
R01A	250	3	1	MD	151.96	-
R01A	250	3	2	MD	163.04	-
R01A	250	3	3	MD	143.90	-
R01A	250	8	1	MD	129.49	-

R01A	250	8	2	MD	137.01	-
R01A	250	8	3	MD	130.71	-
R01A	250	15	1	MD	105.21	-
R01A	250	15	2	MD	98.36	-
R01A	250	15	3	MD	95.52	-
R01A	250	22	1	MD	74.13	-
R01A	250	22	2	MD	83.18	-
R01A	250	22	3	MD	89.76	-
R01A	250	28	1	MD	61.08	-
R01A	250	28	2	MD	68.20	-
R01A	250	28	3	MD	69.54	-
L6S	250	0	1	MD	159.65	-
L6S	250	0	2	MD	168.15	-
L6S	250	0	3	MD	163.37	-
L6S	250	1	1	MD	156.54	-
L6S	250	1	2	MD	159.62	-
L6S	250	1	3	MD	160.00	-
L6S	250	3	1	MD	163.61	-
L6S	250	3	2	MD	173.37	-
L6S	250	3	3	MD	166.81	-
L6S	250	8	1	MD	139.85	-
L6S	250	8	2	MD	138.61	-
L6S	250	8	3	MD	133.23	-
L6S	250	15	1	MD	115.70	-
L6S	250	15	2	MD	118.12	-
L6S	250	15	3	MD	126.82	-
L6S	250	22	1	MD	90.02	-
L6S	250	22	2	MD	95.19	-
L6S	250	22	3	MD	124.73	-
L6S	250	28	1	MD	87.46	-
L6S	250	28	2	MD	86.45	-
L6S	250	28	3	MD	108.43	-
R01A	500	0	1	MD	337.79	-
R01A	500	0	2	MD	333.47	-
R01A	500	0	3	MD	346.72	-
R01A	500	1	1	MD	271.45	-
R01A	500	1	2	MD	261.45	-
R01A	500	1	3	MD	313.73	-
R01A	500	3	1	MD	262.28	-
R01A	500	3	2	MD	246.38	-
R01A	500	3	3	MD	257.68	-
R01A	500	8	1	MD	234.89	-
R01A	500	8	2	MD	223.71	-
R01A	500	8	3	MD	216.20	-
R01A	500	15	1	MD	202.74	-
R01A	500	15	2	MD	203.39	-
R01A	500	15	3	MD	205.02	-
R01A	500	22	1	MD	153.91	-
R01A	500	22	2	MD	151.19	-
R01A	500	22	3	MD	162.77	-
R01A	500	28	1	MD	140.44	-
R01A	500	28	2	MD	141.03	-
R01A	500	28	3	MD	142.05	-
L6S	500	0	1	MD	340.23	-
L6S	500	0	2	MD	360.71	-

L6S	500	0	3	MD	320.03	-
L6S	500	1	1	MD	314.88	-
L6S	500	1	2	MD	308.21	-
L6S	500	1	3	MD	331.95	-
L6S	500	3	1	MD	326.46	-
L6S	500	3	2	MD	319.17	-
L6S	500	3	3	MD	357.56	-
L6S	500	8	1	MD	305.73	-
L6S	500	8	2	MD	313.89	-
L6S	500	8	3	MD	282.39	-
L6S	500	15	1	MD	277.01	-
L6S	500	15	2	MD	283.62	-
L6S	500	15	3	MD	263.33	-
L6S	500	22	1	MD	237.54	-
L6S	500	22	2	MD	254.00	-
L6S	500	22	3	MD	259.47	-
L6S	500	28	1	MD	209.93	-
L6S	500	28	2	MD	192.85	-
L6S	500	28	3	MD	199.40	-
R01A	50	0	1	PD+C	44.95	0.00
R01A	50	0	2	PD+C	47.13	0.00
R01A	50	0	3	PD+C	40.83	0.00
L6S	50	0	1	PD+C	36.13	0.00
L6S	50	0	2	PD+C	36.81	0.00
L6S	50	0	3	PD+C	35.51	0.00
R01A	50	1	1	PD	39.45	0.37
R01A	50	1	2	PD	35.06	0.27
R01A	50	1	3	PD	37.26	0.30
R01A	50	3	1	PD	29.93	1.05
R01A	50	3	2	PD	31.12	0.82
R01A	50	3	3	PD	30.07	0.98
R01A	50	8	1	PD	24.88	1.25
R01A	50	8	2	PD	30.36	3.79
R01A	50	8	3	PD	25.68	1.27
R01A	50	15	1	PD	21.61	0.86
R01A	50	15	2	PD	21.41	2.66
R01A	50	15	3	PD	21.77	1.53
R01A	50	22	1	PD	21.26	0.75
R01A	50	22	2	PD	19.23	0.93
R01A	50	22	3	PD	20.58	1.23
R01A	50	28	1	PD	15.50	0.68
R01A	50	28	2	PD	16.60	0.70
R01A	50	28	3	PD	15.10	1.07
L6S	50	1	1	PD	30.17	0.85
L6S	50	1	2	PD	31.60	0.74
L6S	50	1	3	PD	33.05	0.31
L6S	50	3	1	PD	29.31	0.65
L6S	50	3	2	PD	32.18	1.17
L6S	50	3	3	PD	30.34	0.86
L6S	50	8	1	PD	28.28	1.08
L6S	50	8	2	PD	28.32	1.82
L6S	50	8	3	PD	26.90	2.25
L6S	50	15	1	PD	25.21	1.46
L6S	50	15	2	PD	22.55	3.45
L6S	50	15	3	PD	21.75	1.71

L6S	50	22	1	PD	18.41	2.19
L6S	50	22	2	PD	18.96	7.32
L6S	50	22	3	PD	21.61	1.66
L6S	50	28	1	PD	21.39	1.36
L6S	50	28	2	PD	19.98	1.17
L6S	50	28	3	PD	21.14	1.13
R01A	50	1	1	C	39.19	0.00
R01A	50	1	2	C	40.95	0.00
R01A	50	1	3	C	42.49	0.00
R01A	50	3	1	C	39.31	0.24
R01A	50	3	2	C	36.05	0.22
R01A	50	3	3	C	39.67	0.40
R01A	50	8	1	C	36.60	0.72
R01A	50	8	2	C	36.05	0.38
R01A	50	8	3	C	36.94	0.33
R01A	50	15	1	C	35.60	0.82
R01A	50	15	2	C	33.46	0.80
R01A	50	15	3	C	34.32	0.58
R01A	50	22	1	C	32.77	0.74
R01A	50	22	2	C	32.51	0.24
R01A	50	22	3	C	31.19	0.63
R01A	50	28	1	C	29.34	0.37
R01A	50	28	2	C	31.01	0.50
R01A	50	28	3	C	29.87	0.70
L6S	50	1	1	C	35.19	0.00
L6S	50	1	2	C	34.50	0.00
L6S	50	1	3	C	36.36	0.00
L6S	50	3	1	C	35.89	0.16
L6S	50	3	2	C	33.27	0.06
L6S	50	3	3	C	35.57	0.13
L6S	50	8	1	C	33.91	0.22
L6S	50	8	2	C	32.39	0.86
L6S	50	8	3	C	30.41	0.14
L6S	50	15	1	C	31.84	0.11
L6S	50	15	2	C	31.77	0.32
L6S	50	15	3	C	30.83	0.34
L6S	50	22	1	C	28.37	0.36
L6S	50	22	2	C	25.43	0.52
L6S	50	22	3	C	28.88	0.41
L6S	50	28	1	C	24.64	0.50
L6S	50	28	2	C	29.25	1.12
L6S	50	28	3	C	27.37	0.66

SI-2 Quality criteria and pretests

Soil sampling, preparation and pre-incubation

Soils were sampled by the provider and conditioned to meet the requirements of OECD guide 307. Soil sampling was carried out by Fraunhofer IME (Schmallenberg, Germany) for the "RefeSol 01-A" soil and by LUFA (Speyer, Germany) for the clay soil "LUFA 6S". Soil sampling during or immediately after long periods of drought (> 30 days), frost or flooding was avoided. Soil was prepared (removal of vegetation, larger soil organisms and stones, and sieving through a 2-mm sieve) within one week after sampling. Soil was stored in at 4°C under aerobic conditions for less than 1 month until used for degradation experiments. Before the main experiment was conducted moisture of both soils was adjusted to 40% of maximum water holding capacity to ensure optimal microbial conditions. The moisture-adjusted soils were pre-incubated in the dark at 20°C under aerobic conditions for 1 week to re-establish equilibrium of microbial metabolism following the change from storage conditions to incubation conditions.

Confirmation of the nominal concentration of the AFB1 fortification standard

It was verified that the measured concentration of the stock solution prepared by dissolving 10 mg of crystalline AFB1 in 20 mL of MeCN (see Methods) corresponds to the nominal concentration of 500 mg L^{-1}. For this purpose, 3 aliquots were taken and diluted to a nominal concentration of 5 µg L^{-1} and measured by HPLC-FLD and quantified via external calibration (see Methods). Concentrations of 4.95, 5.14 and 5.10 were determined. The obtained concentrations were checked for significant differences from the nominal concentration of 5 by one-sample t.test. The obtained test statistic confirms that there is no significant difference from the nominal concentration ($t(2) = 1.095$, $p=0.388$).

Glass Adsorption test

It was checked whether the dissipation of AFB1 in the abiotic control(C) over time was at least partly due to adsorption of AFB1 on the glass material. For this purpose, empty glass jars were fortified as for the abiotic degradation samples. Immediately after spiking and evaporation step (t0) and after 8 days (t8) of incubation, the fortified jars were subjected to the same analytical procedure as the soil samples and the concentration was determined via HPLC-FLD using external calibration. Spike recoveries were 94.8 ± 10.4 and 94.2 ± 3.50 for t0 and t8, respectively. No significant decrease in extractable concentration over time was observed ($t(2.4) = 0.1$, $p = 0.928$).

Homogeneity of spike distribution

Table 2. The homogeneous distribution of AFB1 in the sterile (PD+C) and nonsterile (MD) soils fortified with 0.5. 5, 50, 250 and 500 µg kg^{-1} AFB1 was checked by mean spike recoveries and relative standard deviation of spike revovery (in brackets) at day 0 in triplicate.

Soil	Type	AFB1 level (µg kg^{-1})	%-Recovery
R01A	MD	0,5	67(6)
		5	90(4)
		50	71(1)
		250	66(3)
		500	68(2)
L6S	MD	0,5	64(14)
		5	65(3)
		50	66(1)
		250	65(3)
		500	68(6)
R01A	PD+C	50	89(7)
L6S	PD+C	50	72(2)

SI-3 Chromatographic data

LC-HRMS chromatograms and spectra

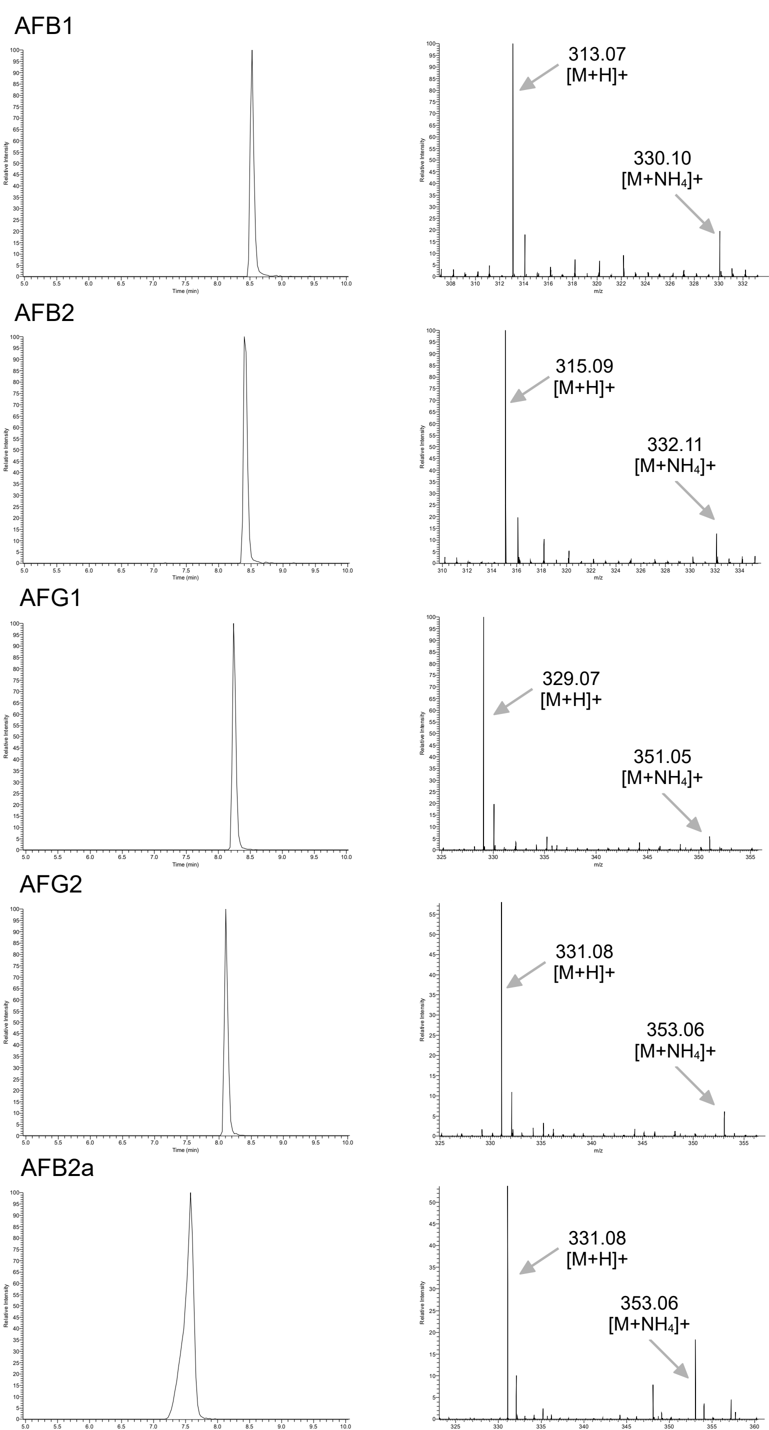

Figure 1. Chromatograms (left) and mass spectra (right) gained from LC-HRMS measurements of AFB1, AFB2, AFG1, AFG2 and AFB2a standards (5 µg L^{-1}).

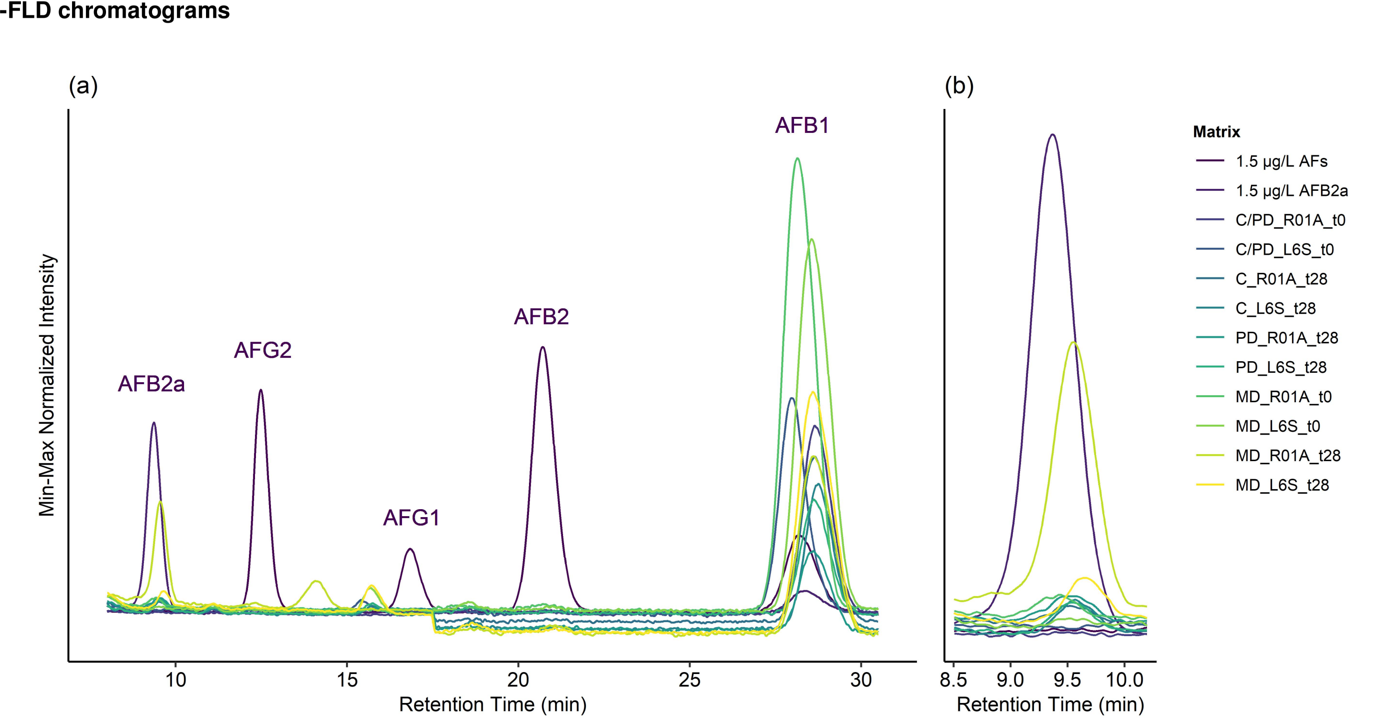

Figure 2. Chromatograms gained from HPLC-FLD measurements showing all investigated AFs i.e. AFB1, AFB2, AFG1, AFG2 and AFB2a Peaks (a) and a close up for AFB2a (b) in standards and extracts from day 0 and day 28. Extracts were obtained from sterile (PD+C) and nonsterile (MD) incubated soils fortified with 50 µg kg^{-1} AFB1.

SI-4 Statistical analyses

Statistical tests for the effect of incubation conditions on AFB1 dissipation

Table 3. ANOVA model summary: Effect of degradation type (MD, PD, C) and soil (sandy loam, clay) on AFB1 dissipation in terms of normalized AFB1 concentration at the end of 28-days incubation.

Predictor	DF_{effect}	DF_{error}	F	p
Degradation Type	2	12	72.15	<0.001
Soil	1	12	70.999	<0.001
Degradation Type:Soil	2	12	5.787	0.017

Table 4. Post-hoc test: Effect of the degradation type (MD, PD, C) for the individual soils (sandy loam, clay) on AFB1 dissipation in terms of normalized AFB1 concentration at the end of 28-days incubation.

Soil	DF_{effect}	DF_{error}	F	p
L6S	2	12	18.9	<0.001
R01A	2	12	59.1	<0.001

Table 5. Post-hoc test: Effect of the soil type (sandy loam, clay) for the individual degradation types (MD, PD, C) on AFB1 dissipation in terms of normalized AFB1 concentration at the end of 28-days incubation.

Degradation type	DF_{effect}	DF_{error}	F	p
C	1	12	4.7	0.051
MD	1	12	31.7	<0.001
PD	1	12	46.2	<0.001

Statistical tests for the effect of AFB1 initial concentration AFB1 dissipation due to microbial degradation

Table 6. Multiple regression summary: Effect of initial AFB1 level (0.5, 5, 50, 250, 500 µg kg^{-1}) and soil type (sandy loam, clay) on AFB1 dissipation in terms of normalized AFB1 concentration at the end of 28-days incubation due to microbial degradation.

Predictor	Estimate	t	p
(Intercept)	0.64 ± 0.02	39.2	<0.001
Initial AFB1 level	$(-1.41 \pm 0.65) \times 10^{-4}$	-2.2	0.040
Soil	-0.28 ± 0.02	-12.0	<0.001
Initial AFB1 level:Soil	$(2.61 \pm 0.92) \times 10^{-4}$	2.8	0.009

Table 7. Post-hoc test: Effect of the initial AFB1 level (0.5, 5, 50, 250, 500 µg kg^{-1}) for the individual soils (sandy loam, clay) on AFB1 dissipation in terms of normalized AFB1 concentration at the end of 28-days incubation.

Soil	DF_{effect}	DF_{error}	F	p
L6S	1	26	4.7	0.04
R01A	1	26	3.5	0.074

Table 8. Post-hoc test: Effect of soil type (sandy loam, clay) for the individual initial AFB1 levels (0.5, 5, 50, 250, 500 μkg^{-1}) on AFB1 dissipation in terms of normalized AFB1 concentration at the end of 28-days incubation.

AFB1 level (μg kg^{-1})	DF_{effect}	DF_{error}	F	p
0.5	1	26	72.6	<0.001
5	1	26	60.9	<0.001
50	1	26	21.5	<0.001
250	1	26	19.1	<0.001
500	1	26	19.3	<0.001

8.5 Supporting Information on Chapter 5

Appendix A. Calibration Figures

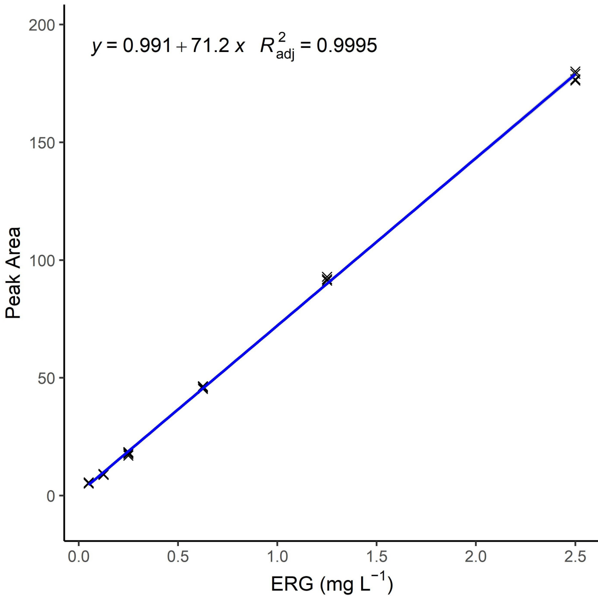

Figure A1. Calibration curve for ergosterol.

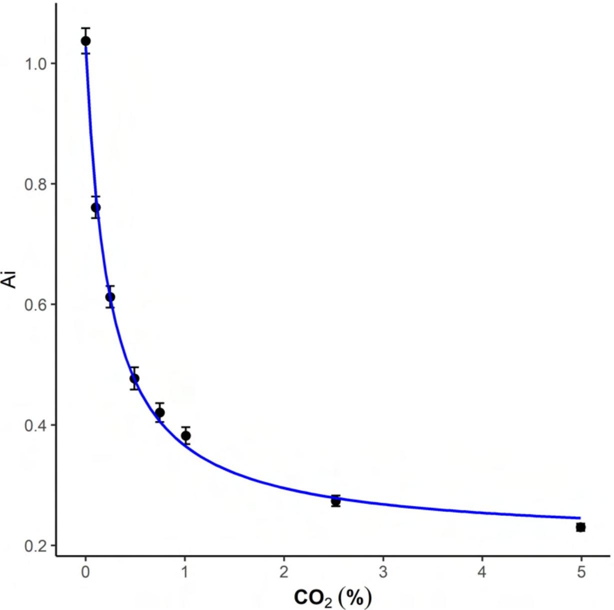

Figure A2. Nonlinear calibration curve for MicroResp. The x axis shows the percentage air fraction of CO_2 and the y axis shows the normalized absorbance at 572 nm.

Appendix B. Microbial Responses to Aflatoxin B1

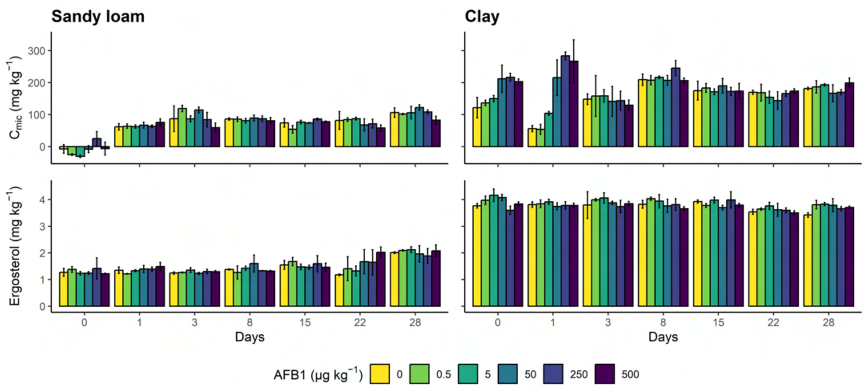

Figure A3. Microbial and fungal biomass: Barplots showing the average values for microbial biomass carbon (C_{mic}) and ergosterol as bioindicators for fungal biomass (ERG). The error bars represent the standard deviations.

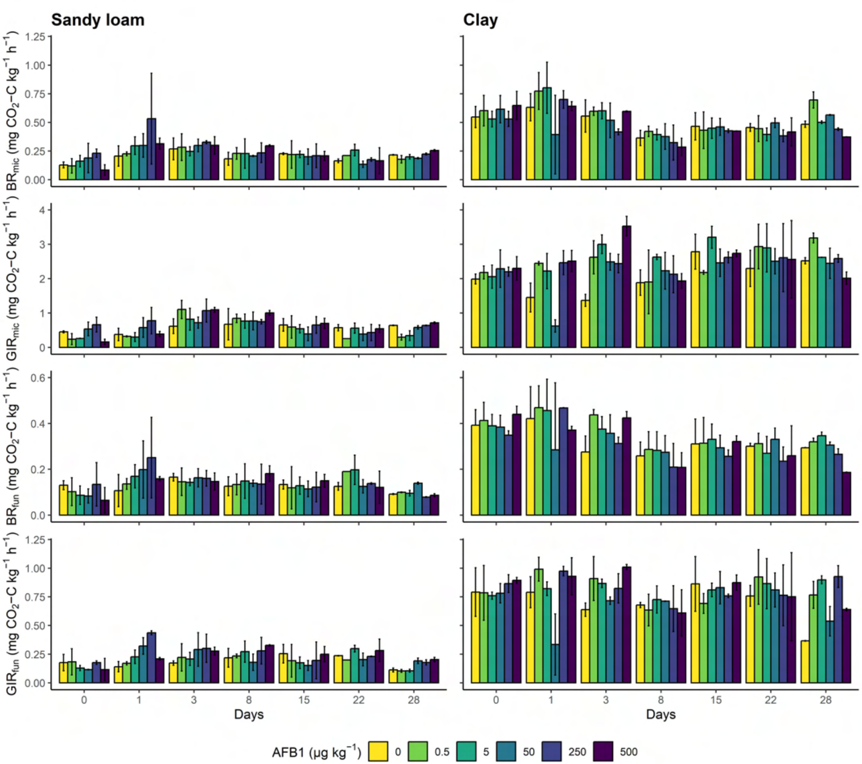

Figure A4. Microbial and fungal activities: Barplots showing mean values for microbial basal respiration (BR_{mic}), microbial glucose-induced respiration (GIR_{mic}), fungal basal respiration (BR_{fun}), and fungal glucose-induced respiration (GIR_{fun}). The error bars represent the standard deviations.

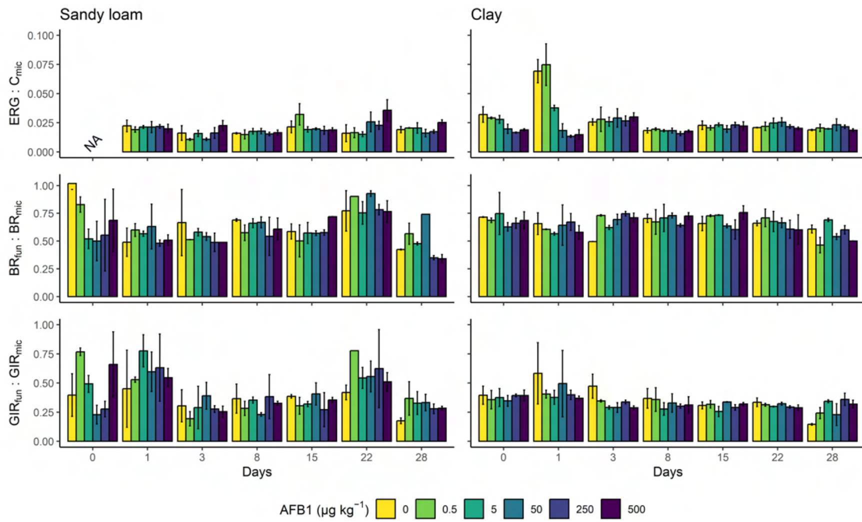

Figure A5. Biomass and activity ratios: Fungal-to-microbial ratios for the biomass (ERG:C_{mic}), basal respiration (BR_{fun}:BR_{mic}), and glucose-induced respiration (GIR_{fun}:GIR_{mic}).

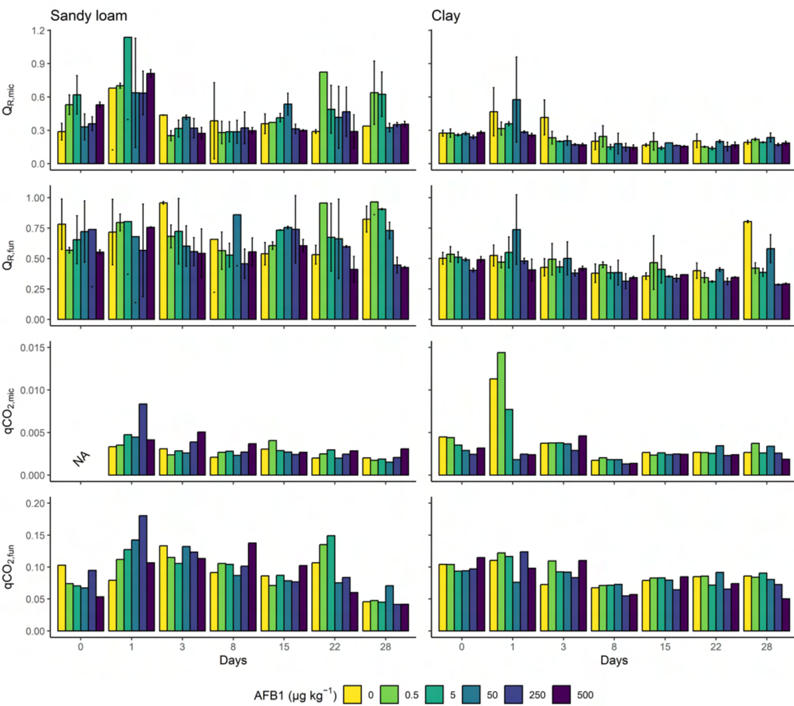

Figure A6. Ecophysiological ratios: Microbial basal-to-substrate induced respiration ($Q_{R,mic}$), fungal basal-to-substrate induced respiration ($Q_{R,fun}$), microbial metabolic quotient ($qCO_{2,mic}$), and fungal metabolic quotient ($qCO_{2,fun}$).

Appendix C. Principal Component Analysis

PRC is a constrained ordination technique and a special case of redundancy analysis (RDA). A problem with traditional ordination methods such as RDA is that temporal changes in treatment effects make treatment effects (e.g., compared to a control) difficult to determine if time does not follow a straight line in the ordination graph, resulting in a cluttered and difficult-to-interpret ordination graph [60,61]. PRC overcomes this problem by focusing on the differences between the species (e.g., the respiration rates induced by the individual substrates) compositions of treatments at each sampling date [62]. For PRC construction, an RDA model is fitted to the multivariate response using treatment, time, and their interaction as predictors. Since the main interest is in the multivariate response due to treatment and not due to overall temporal change, the main effect of time is factored out and only the treatment:time interaction is kept. As a result, the RDA axes show only the change explained by treatment and the treatment:time interaction, but not the overall temporal trend [63]. The PRC plot shows on the y axis the difference in the canonical coefficient of the treatments (i.e., the individual AFB1 concentration levels) from the nonspiked control (represented graphically as a zero line), and on the x axis the incubation time. The further the communities are from the control line, the more they differ from the control group. The extraction of the accompanying species (i.e., substrates) weights allows interpretation at the species level. The higher the respective species weight, the more likely that the actual response pattern of the species follows the pattern in the PRC, while species with a highly negative weight are assumed to show the opposite pattern. Species with a weighting close to zero either show no response or a response that does not match the pattern shown by the PRC [61].

Table A1. Principal response curves: Differences in the canonical coefficients of the treatments (i.e., the individual AFB1 concentration levels) from the nonspiked control for both soils (sandy loam and clay) and systems (microbial and fungal) over 28 days of incubation.

Day	AFB1 (μg kg^{-1})	Sandy Loam		Clay	
		Microbial	Fungal	Microbial	Fungal
0	0.5	0.048	0.086	0.034	0.04
	5	0.022	0.179	−0.013	0.007
	50	−0.042	0.204	0.067	−0.007
	250	−0.135	0.006	0.059	0.046
	500	0.104	0.233	0.124	0.15
1	0.5	0.073	−0.007	0.298	0.201
	5	0.016	−0.219	0.211	0.075
	50	−0.025	−0.278	−0.308	−0.4
	250	−0.468	−0.667	0.258	0.157
	500	0.029	−0.138	0.254	0.065
3	0.5	−0.039	0.012	0.331	0.316
	5	0.101	0.051	0.409	0.185
	50	0.146	0.002	0.248	0.106
	250	−0.073	−0.083	0.201	0.081
	500	0.072	0.019	0.545	0.329
8	0.5	−0.087	0.018	0.039	0.019
	5	−0.139	−0.076	0.19	0.104
	50	0.014	0.038	0.088	0.049
	250	−0.048	−0.019	0.047	−0.057
	500	−0.194	−0.195	−0.024	−0.082
15	0.5	0.073	0.085	−0.191	−0.108
	5	0.068	0.087	0.084	−0.006
	50	0.143	0.102	−0.099	−0.061
	250	0.014	0.076	−0.063	−0.094
	500	0.008	−0.013	−0.026	0.009
22	0.5	0.058	−0.071	0.158	0.068
	5	−0.052	−0.179	0.145	0.038
	50	0.046	0.046	0.067	0.043
	250	0.004	0.033	0.062	−0.071
	500	−0.017	0.01	0.049	−0.059
28	0.5	0.22	−0.046	0.23	0.224
	5	0.181	−0.057	0.019	0.343
	50	0.082	−0.232	0.003	0.072
	250	−0.005	−0.091	0.027	0.256
	500	−0.046	−0.145	−0.134	0.007

Table A2. Species weight for the principal response curves. Species represent the carbon substrates used for substrate-induced respiration measurements. Glu = D-glucose, Gal = D-galactose, GABA = γ-aminobutyric acid, NAGA = N-acetylglucosamine, NaCit = sodium citrate, and aCD = α-cyclodextrin.

Substrate	Sandy Loam		Clay	
	Microbial	Fungal	Microbial	Fungal
aCD	−0.77	−0.5	0.32	0.35
Ala	−0.79	−0.32	0.62	0.37
GABA	−0.32	−0.22	0.46	0.31
Gal	−0.42	−0.28	0.53	0.36
Glu	−0.68	−0.44	1.75	0.88
NaCit	−0.45	−0.32	0.74	0.34
NAGA	−0.39	−0.27	0.41	0.31
W	−0.24	−0.22	0.22	0.27

Appendix D. Test Statistics of the Multiple Regression Models

Table A3. Test statistics of the multiple regression models used to evaluate the effect of AFB1 concentration, incubation time and their interaction on soil microbial and ecophysiological parameters. Significant results ($p < 0.05$) are shown in bold.

Response	Soil	Predictor	B	SE	t	*p*
C_{mic}	clay	Intercept	151	7.48	20.173	**<0.001**
		Time	1.2	0.5	2.41	**0.017**
		AFB1	0.147	0.0327	4.516	**<0.001**
		Time:AFB1	−0.00642	0.00218	−2.942	**0.004**
	sandy loam	Intercept	76.7	3.74	20.513	**<0.001**
		Time	0.703	0.231	3.038	**0.003**
		AFB1	−0.0119	0.0163	−0.731	0.466
		Time:AFB1	−0.00082	0.00101	−0.813	0.418
ERG	clay	Intercept	3.93	0.0303	129.616	**<0.001**
		Time	−0.00894	0.00203	−4.411	**<0.001**
		AFB1	−0.00035	0.000132	−2.644	**0.009**
		Time:AFB1	9.3×10^{-6}	8.85×10^{-6}	1.051	0.296
	sandy loam	Intercept	0.788	0.0154	51.069	**<0.001**
		Time	−0.00732	0.00103	−7.097	**<0.001**
		AFB1	-1.85×10^{-5}	6.74×10^{-5}	−0.274	0.784
		Time:AFB1	-5.43×10^{-6}	4.5×10^{-6}	−1.205	0.231
BR_{mic}	clay	Intercept	0.552	0.0265	20.866	**<0.001**
		Time	−0.00301	0.00177	−1.704	0.092
		AFB1	1.2×10^{-5}	0.000115	0.104	0.918
		Time:AFB1	-1.08×10^{-5}	7.72×10^{-6}	−1.395	0.167
	sandy loam	Intercept	0.235	0.0189	12.428	**<0.001**
		Time	−0.00151	0.00128	−1.18	0.241
		AFB1	9.73×10^{-5}	8.26×10^{-5}	1.178	0.242
		Time:AFB1	−2.66e−06	5.54e−06	−0.48	0.632
BR_{fun}	clay	Intercept	0.37	0.0161	22.894	**<0.001**
		Time	−0.00294	0.00108	−2.722	**0.008**
		AFB1	2.28×10^{-5}	7.05×10^{-5}	0.323	0.748
		Time:AFB1	8.17×10^{-6}	4.71×10^{-6}	−1.735	0.087
	sandy loam	Intercept	0.141	0.0106	13.315	**<0.001**
		Time	−0.00065	0.000714	−0.91	0.366
		AFB1	3.02×10^{-5}	4.61×10^{-5}	0.654	0.515
		Time:AFB1	-2.34×10^{-6}	3.1×10^{-6}	−0.757	0.451
GIR_{mic}	clay	Intercept	2.02	0.109	18.577	**<0.001**
		Time	0.0292	0.00726	4.026	**<0.001**
		AFB1	0.00126	0.000474	2.666	**0.009**
		Time:AFB1	-8.49×10^{-5}	3.17×10^{-5}	−2.682	**0.009**
	sandy loam	Intercept	0.591	0.0533	11.09	**<0.001**
		Time	−0.00327	0.0036	−0.909	0.366
		AFB1	0.000214	0.000233	0.922	0.359
		Time:AFB1	6.93×10^{-6}	1.56×10^{-5}	0.444	0.658
GIR_{fun}	clay	Intercept	0.765	0.0349	21.905	**<0.001**
		Time	−0.000799	0.00234	−0.342	0.733
		AFB1	0.000271	0.000152	1.776	0.08
		Time:AFB1	-1.24×10^{-5}	1.02×10^{-5}	−1.214	0.228
	sandy loam	Intercept	0.215	0.0166	12.897	**<0.001**
		Time	−0.00152	0.00112	−1.357	0.179
		AFB1	8.7×10^{-5}	7.26×10^{-5}	1.199	0.234
		Time:AFB1	2.28×10^{-6}	4.87×10^{-6}	0.468	0.641

Table A3. *Cont.*

Response	Soil	Predictor	B	SE	t	*p*
ERG:C_{mic}	clay	Intercept	38.4	1.98	19.414	**<0.001**
		Time	0.42	0.132	3.182	**0.002**
		AFB1	0.0428	0.00862	4.964	**<0.001**
		Time:AFB1	−0.00178	0.000576	−3.097	**0.002**
	sandy loam	Intercept	0.0176	0.0011	15.998	**<0.001**
		Time	5.56×10^{-5}	6.81×10^{-5}	0.817	0.416
		AFB1	1.64×10^{-6}	4.8×10^{-6}	0.342	0.733
		Time:AFB1	5×10^{-7}	2.97×10^{-7}	1.684	0.095
BR_{fun}:BR_{mic}	clay	Intercept	0.671	0.0166	40.54	**<0.001**
		Time	−0.00138	0.00111	−1.251	0.215
		AFB1	-5.59×10^{-5}	7.23×10^{-5}	0.773	0.442
		Time:AFB1	-6.45×10^{-6}	4.83×10^{-6}	−1.335	0.186
	sandy loam	Intercept	0.623	0.0329	18.919	**<0.001**
		Time	0.00032	0.00223	0.144	0.886
		AFB1	−0.000109	0.000144	−0.755	0.452
		Time:AFB1	-1.57×10^{-6}	9.64×10^{-6}	−0.163	0.871
GIR_{fun}:GIR_{mic}	clay	Intercept	0.397	0.0159	24.961	**<0.001**
		Time	−0.00513	0.00106	−4.825	**<0.001**
		AFB1	−0.000111	6.94e−05	−1.595	0.115
		Time:AFB1	7.82×10^{-6}	4.64×10^{-6}	1.685	0.096
	sandy loam	Intercept	0.432	0.0362	11.932	**<0.001**
		Time	−0.00255	0.00244	−1.041	0.301
		AFB1	3.5e−05	0.000158	0.222	0.825
		Time:AFB1	-1.95×10^{-6}	1.06×10^{-5}	−0.184	0.855
BR_{mic}:GIR_{mic}	clay	Intercept	0.3	0.019	15.814	**<0.001**
		Time	−0.00524	0.00127	−4.125	**<0.001**
		AFB1	−0.000183	8.29×10^{-5}	−2.203	**0.03**
		Time:AFB1	6.16×10^{-6}	-5.54×10^{-6}	1.112	0.27
	sandy loam	Intercept	0.483	0.0466	10.361	**<0.001**
		Time	−0.00174	0.00315	−0.554	0.581
		AFB1	-1.38×10^{-5}	0.000203	−0.068	0.946
		Time:AFB1	-1.22×10^{-5}	1.36×10^{-5}	−0.896	0.373
BR_{fun}:GIR_{fun}	clay	Intercept	0.48	0.0223	21.585	**<0.001**
		Time	−0.00181	0.00149	−1.214	0.228
		AFB1	−0.000115	9.71×10^{-5}	−1.187	0.239
		Time:AFB1	-7.87×10^{-6}	6.49×10^{-6}	−1.212	0.229
	sandy loam	Intercept	0.687	0.039	17.609	**<0.001**
		Time	0.0027	0.00264	1.023	0.31
		AFB1	−0.000132	0.00017	−0.777	0.439
		Time:AFB1	-2.15×10^{-5}	1.14×10^{-5}	−1.88	0.064
$qCO_{2,mic}$	clay	Intercept	−5.55	0.125	−44.546	**<0.001**
		Time	−0.0164	0.00833	−1.972	0.056
		AFB1	−0.000966	0.000544	−1.777	0.084
		Time:AFB1	2.03×10^{-5}	3.63×10^{-5}	0.559	0.58
	sandy loam	Intercept	0.00358	0.00033	10.85	**<0.001**
		Time	-6.1×10^{-5}	2.04×10^{-5}	−2.987	**0.005**
		AFB1	3.01×10^{-6}	1.44×10^{-6}	2.089	**0.045**
		Time:AFB1	-8.52×10^{-8}	8.9×10^{-8}	−0.957	0.346
$qCO_{2,fun}$	clay	Intercept	0.0941	0.00426	22.092	**<0.001**
		Time	−0.000556	0.000285	−1.955	0.058
		AFB1	1.45×10^{-5}	1.86×10^{-5}	0.781	0.44
		Time:AFB1	-2.46×10^{-6}	1.24×10^{-6}	−1.984	0.055
	sandy loam	Intercept	0.109	0.00814	13.389	**<0.001**
		Time	−0.00122	0.000544	−2.244	**0.031**
		AFB1	1.53×10^{-5}	3.55×10^{-5}	0.429	0.67
		Time:AFB1	-2.19×10^{-6}	2.38×10^{-6}	−0.923	0.362

Appendix E. Test Statistics of the Monte Carlo Permutation Test

Table A4. Test statistics of the Monte Carlo permutation test used to assess the significance of the effects of AFB1 concentration on the multivariate response (canonical coefficient of PRC). Significant results ($p < 0.05$) are shown in bold.

Fraction	Soil	Day	DF	F	*p*
Microbial	sandy loam	0	1	0.26	0.838
		1	1	1.15	0.382
		3	1	2.36	0.105
		8	1	1.48	0.209
		15	1	0.13	0.838
		22	1	0.24	0.846
		28	1	2.7	0.117
	clay	0	1	1.51	0.21
		1	1	0.32	0.689
		3	1	5.36	**0.03**
		8	1	0.89	0.397
		15	1	0.03	0.997
		22	1	0.07	0.932
		28	1	3.71	0.064
Fungal	sandy loam	0	1	1.05	0.317
		1	1	2.4	0.109
		3	1	2.03	0.119
		8	1	1.08	0.324
		15	1	0.04	0.958
		22	1	1.13	0.314
		28	1	5.43	**0.013**
	clay	0	1	0.65	0.523
		1	1	0.11	0.911
		3	1	1.08	0.296
		8	1	1.06	0.344
		15	1	0.12	0.935
		22	1	0.56	0.479
		28	1	2.43	0.118

Appendix F. Dissolved Organic Matter in Nonfumigated Samples

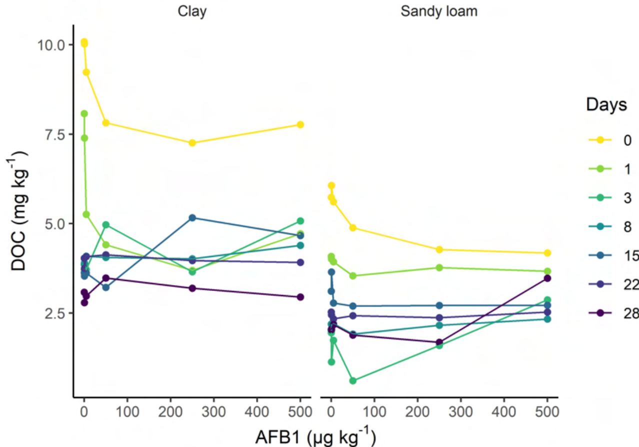

Figure A7. Dissolved organic carbon in the nonfumigated sandy loam and clay soils as a function of AFB1 concentration.

8.6 List of Figures

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8.8 List of Abbreviations

ΣAFs	Sum of aflatoxins (AFB1+AFB2+AFG1+AFG2)
(q)PCR	(quantitative) polymerase chain reaction
ACE	Acetone
ACOH	Acetic acid
AF(s)	Aflatoxin(s)
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFB2a	Aflatoxin B2a
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AGC	Automatic gain control
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
ARSO	African Standards Organization
CAC	Codex Alimentarius Commission
C	Control
Corg	Organic carbon content
CFU	Colony forming units
dw	Dry weight
EC	European Commission
EAC	East African Community
EACU	Eurasian Customs Union
ESI	Electrospray ionization
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
GABA	γ-aminobutyric acid
GAL	D-galactose
GIR	Glucose induced respiration
GIR_{fun}	Fungal glucose induced respiration

GIR_{mic}	Microbial glucose induced respiration
GSO	Gulf Cooperation Council Standardization Organization
HBA	Hydrogen Bond Acceptor Count
HBD	Hydrogen Bond Donor Count
HPLC-FLD	High-performance liquid chromatography with fluorescence detection
IAC	Immunoaffinity chromatography
LC-MS	Liquid chromatography-mass spectrometry
$Log(c_{max,w})$	Water solubility, logarithmic scale
$Log(K_H)$	Henry coefficient, logarithmic scale
$Log(K_{OA})$	Octanol-Air-partitioning coefficient, logarithmic scale
$Log(K_{OC})$	Soil absorption coefficient, logarithmic scale
$Log(K_{OW})$	Octanol-Water-partitioning coefficient, logarithmic scale
$Log(P_v)$	Vapor pressure, logarithmic scale
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar mass
MAD	Median absolute deviation
MD	Microbial degradation
MERCOSUR	Mercado Común del Sur (Southern Common Market)
MeACN	Acetonitrile
MeOH	Methanol
MMC	Matrix-effect calibration
NAGA	N-acetylglucosamine
NaCit	Sodium citrate
PLFA	Phospholipid Fatty-Acid-Analysis
PRC	Principal response curves
qCO2	Metabolic quotient
Q_R	Basal-to-substrate induced respiration ratio
Q_{Rfun}	Fungal basal-to-substrate induced respiration ratio
Q_{Rmic}	Microbial basal-to-substrate induced respiration ratio
SFE	Supercritical-fluid-extraction

SLE	Solid-liquid-extraction
SIDA	Stable isotope dilution assays
SIR	Substrate induced respiration
SIR_{fun}	Fungal substrate induced respiration
SIR_{mic}	Microbial substrate induced respiration
SPE	Solid-phase extraction
T_b	Boiling point
TOC	Total organic carbon content
T_m	Melting point
UV	Ultraviolet radiation
USE	Ultrasonication-assisted solvent extraction
VIF	Variance inflation factor
WHO	World Health Organization
WHC	Water holding capacity

8.9 Curriculum Vitae

Julius Albert — Curriculum Vitae

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Education

since 01/2019 — **PhD, Environmental Sciences**
University of Kaiserslautern–Landau (RPTU), Landau, Germany
Thesis title: "Aflatoxins in the Soil Environment – Occurrence, Fate and Consequences for the Soil Microbiome and Associated Functions."

10/2015 – 12/2018 — **M.Sc., Environmental Sciences (Final grade: 1.4)**
University of Koblenz–Landau (UKL), Landau, Germany
Thesis title: "Combined Strategy to Remediate a Lindane Polluted Soil with the White Rot Fungus *Pleurotus ostreatus* & Biochar." (Grade: 1.3)

10/2011 – 02/2016 — **B.Sc., Environmental Sciences (Final grade: 2.2)**
University of Koblenz–Landau (UKL), Landau, Germany
Thesis title: "Auswirkungen von HTC-Biokohle auf die Mikrobielle Aktivität und Regenwürmer im Feldversuch." (Grade: 1.0)

Research Experience

11/2022 – 09/2023 — **Research Associate and Project Management**
RLP AgroSciences GmbH, Neustadt / Weinstr., Germany
– Collaboration on the MycoPom project: "Development of a Fungal Preparation Based on Grape Marc for the Biological Remediation of Organically Polluted Soils".
– Preparation of research proposals.
– Participation in project-related conferences.

01/2019 – 09/2023 — **Research Associate**
University of Kaiserslautern–Landau (RPTU), Landau, Germany
– Collaboration on the AflaZ project: "Zero Aflatoxin – A Multidisciplinary Cooperation Project Between German and African Research Institutions".
– Publication of research results with participation in (inter-)national conferences.
– Supervision of student theses and teaching in interdisciplinary research, environmental analysis, environmental chemistry, and ecology.

11/2018 – 12/2018 — **Student Employee**
University of Kaiserslautern–Landau (RPTU), Landau, Germany
– Support in the organization of workshops.
– Coordination and procurement of orders.

02/2018 – 03/2018 **Intern**
RLP AgroSciences GmbH, Neustadt / Weinstr., Germany
– Sampling, extraction and analysis as part of the plant protection product approval process.

04/2017 – 08/2017 **Intern**
University of Kaiserslautern–Landau (RPTU), Landau, Germany
– Participation in the SOIL-PLAST project by performing respiration analyses on soil samples and contributing to a publication.

04/2015 – 06/2015 **Student Employee**
RLP AgroSciences GmbH, Neustadt / Weinstr., Germany
– Development and optimization of the production of pellets from HTC biochar and implementation of quality control of the pellets.

Teaching Experience

since 04/2021 **Ecological Chemistry – Seminar**
University of Kaiserslautern–Landau (RPTU), Landau, Germany
– Co–Lecturer

01/2019 - 03/2022 **Laborübungen Umweltanalytik – Lab exercise**
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– Co–Supervisor

04/2021 - 10/2021 **Messung von Umweltparametern – Field exercise**
University of Kaiserslautern–Landau (RPTU), Landau, Germany
– Co–Supervisor

10/2019 - 03/2021 **Interdisciplinary Case Study / Interdisziplinäre Fallstudie – Project seminar**
University of Kaiserslautern–Landau (RPTU), Landau, Germany
– Co–Supervisor

10/2019 - 03/2020 **Projekt Organische Chemie – Project seminar**
University of Kaiserslautern–Landau (RPTU), Landau, Germany
– Co-Supervisor

(Co-)supervision of final theses

10/2022 **Selina Morscheid, B.Sc. Mensch und Umwelt**
University of Koblenz–Landau (UKL), Landau, Germany
Thesis title: "Development of energy-saving disinfection methods for agro-residues for the cultivation of *Pleurotus ostreatus*"

09/2021 **Sarah Morlock, B.Sc. Environmental Science**
University of Koblenz–Landau (UKL), Landau, Germany
Thesis title: "Auswirkung verschiedener Hydrogelapplikationstechniken

unter Nutzung von Xanthan auf Wachstum und trockenheits- und transplantationsschockbedingte Mortalität von Jungbäumen (*Castanea sativa* Mill.) im Pfälzerwald"

09/2021 **Kay Seufferheld, B.Sc. Environmental Science**
University of Koblenz–Landau (UKL), Landau, Germany
Thesis title: "Auswirkungen unterschiedlicher Konzentrationen von Hydrogelen in sandigem Boden auf das Maiswachstum (*Zea mays convar. saccharata*) unter verschiedenen Trockenszenarien"

06/2021 **Katharina Hanten, B.Ed. Lehramt Biologie und Chemie**
University of Koblenz–Landau (UKL), Landau, Germany
Thesis title: "Quantitative Bestimmung von Ergosterol (HPLC-UV) als Indikator für Pilzbiomasse Zusammenhang mit Bodenparametern am Beispiel von Maisfeldern"

04/2021 **Maureen Roth, M.Ed. Lehramt Biologie und Chemie**
University of Koblenz–Landau (UKL), Landau, Germany
Thesis title: "Auftreten von Mykotoxinen in Böden während der Maiswachstumsphase unter Berücksichtigung physikochemischer, klimatischer und mikrobieller Bodenparameter"

01/2021 **Camilla More, M.Sc. Ecotoxicology**
University of Koblenz–Landau (UKL), Landau, Germany
Thesis title: "Effects of synthetic and biogenic hydrogels on soil microbial activity and biomass under two contrasting soil moisture conditions"

07/2020 **Niklaus Dahlke, B.Sc. Environmental Science**
University of Koblenz–Landau (UKL), Landau, Germany
Thesis title: "Validierung einer analytischen Methode zur Bestimmung von Aflatoxinen (B1, B2, G1, G2) in landwirtschaftlichen Böden mittels HPLC-FLD"

04/2020 **Henri Kurz, B.Ed. Lehramt Chemie und Französisch**
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Thesis title: "Fluoreszenzverstärkung von Mykotoxinen durch photochemische Derivatisierung und ihr Abbau in verschiedenen Medien"

09/2019 **Camilla More, B.Ed. Lehramt Chemie und Geographie**
University of Koblenz–Landau (UKL), Landau, Germany
Thesis title: "Entwicklung einer analytischen Methode zur Bestimmung von Aflatoxinen (B1, B2, G1, G2) in landwirtschaftlichen Böden mittels HPLC-FLD"

Continuing Education

since 10/2023 **Data Science Bootcamp (16 weeks full time)**
Data Science Institute, Berlin, Germany (Remote)

04/2018 – 02/2023 **Specialist Consultant for Mycology**
German Mycological Society (DGfM), Karlsruhe, Germany
Thesis title: "Ermittlung des Potenzials eines Enzymextraktes aus verbrauchtem Pilzkompost von *Pleurotus ostreatus* zum Abbau von Pharmazeutika in wässrigen Medien."

01/2021 – 12/2022 **Data Analyst in R**
DataCamp Inc., New York, USA (Remote)

05/2022 **Microscopy of the fungi**
Schwarzwälder Pilzlehrschau, Hornberg, Germany

01/2022 **Creating Reports and Digital Apps for Dynamically Changing Data Using R-Studio and the Knitr and Shiny Packages**
Interdisciplinary Graduate Center (IPZ), Landau, Germany

10/2021 **Metabolomics workshop: Analysis of mycotoxin metabolites in soil extracts using liquid chromatography with time-of-flight mass spectrometer (LC-TOF) and bioinformatics software**
Max Rubner-Institut (MRI), Detmold, Germany

06/2021 **Introduction to LaTeX**
Interdisciplinary Graduate Center (IPZ), Landau, Germany

11/2018 **IFG Workshop: In search for common ground to realize interdisciplinarity in environmental research - A workshop for interdisciplinary researchers**
Interdisciplinary Graduate Center (IPZ), Landau, Germany

Stays Abroad

10/2022 **Nairobi und Kisumu, Kenya**
– Knowledge transfer & networking (AflaZ project)
– Presentation of research results at conferences

11/2022 - 12/2022 **Nairobi, Kenya**
– Knowledge transfer & networking (AflaZ project)
– Ambassadorial Activity: Establishing a Collaboration between the University of Kaiserslautern-Landau and the University of Nairobi.
– Preparation of field sampling and laboratory analysis as part of the AflaZ project

Fellowships

since 03/2021 German Soil Science Society (DBG), Berlin, Germany

since 09/2018 German Mycological Society (DGfM), Karlsruhe, Germany

Awards

07/2019	Research Award of the Department 7 (Natural and Environmental Sciences) for the best Master's thesis in in a scientific discipline *University of Koblenz–Landau (UKL), Landau, Germany*

Skills

Languages	**German:** Native **English:** Proficient (10 school years, international teamwork, studying, lecturing, and publishing/presenting in english language) **French:** Basics (5 school years)
Analytics	**HPLC:** Proficient in HPLC-UV/VIS, HPLC-FLD & LC-HRMS **GC:** Advanced in GC-FID & GC-MS **Radiochemistry:** Basics in HPLC-Radio & LSC **Spectroscopy:** Advanced in UV/VIS- & Fluorescence spectroscopy **Microbiology:** Proficient in Respirometry (OxiTop, CarboBot & MicroResp), Chloroform-Fumigation-Extraction method, Phospholipid-derived fatty acids analysis, Cell culture & fermentation **Molecular Genetics:** Basics in PCR, Sanger sequencing, phylogeny reconstruction, sequence editing via Geneious, sequence alignment via MEGA
Computational Skills	**Office applications:** Proficient in Libre/MS **Operating Systems:** Advanced in Linux (Manjaro) & Windows (98 – 12), basics in Apple operating systems **Writing and formatting:** Advanced in Word, LaTeX & Authorea **Data analysis/science & Machine Learning:** Proficient in R and Excel, advanced in Python & MySQL **Visualization:** Proficient in R (e.g. ggplot), advanced in Python (e.g. seaborn, matplotlib) and Tableau **Programming languages:** Proficient in R, advanced in TeX, advanced in Python & SQL **GIS:** Basics in R, QGIS, & GRASS for spatial data analysis & visualization **Artificial intelligence:** Basics in OpenAI tools (ChatGPT, DALL-E)

Publications

Peer reviewed articles

- **Albert, J.**, More, C., Korz, S., & Muñoz, K. (2023). Soil Microbial Responses to Aflatoxin Exposure: Consequences for Biomass, Activity and Catabolic Functionality. Soil Systems, 7(1), 23.
- **Albert, J.**, & Muñoz, K. (2022). Kinetics of microbial and photochemical degradation of aflatoxin B1 in a sandy loam and clay soil. Scientific Reports, 12(1), 16849.
- Kenngott, K. G., **Albert, J.**, Meyer-Wolfarth, F., Schaumann, G. & E., Muñoz, K. (2022). Fusarium Mycotoxins in Maize Field Soils: Method Validation and Implications for Sampling Strategy. Toxins,

14(2), 130.

- **Albert, J.**, More, C. A., Dahlke, N. R., Steinmetz, Z., Schaumann, G. E., & Muñoz, K. (2021). Validation of a Simple and Reliable Method for the Determination of Aflatoxins in Soil and Food Matrices. ACS omega, 6(29), 18684-18693.
- Steinmetz, Z., **Albert, J.** & Kenngott, K. (2020). Envalysis: Miscellaneous Functions for Environmental Analyses. University of Koblenz-Landau, Institute for Environmental Sciences, Landau. https://doi.org/ft9p
- Schirmel, J., **Albert, J.**, Kurtz, M. P., & Muñoz, K. (2018). Plasticulture changes soil invertebrate assemblages of strawberry fields and decreases diversity and soil microbial activity. Applied Soil Ecology, 124, 379-393.

Conferences

- **Albert, J.** (2022). Aflatoxins and soil quality (oral presentation). AflaZ Congress 2022, Nairobi, Kenya.
- **Albert, J.** & Muñoz, K. (2022). Contribution of soil properties to the biotic and abiotic degradation processes of aflatoxin B1 (poster). World Congress of Soil Science 2022, Glasgow, Scottland.
- **Albert, J.**, & Muñoz, K. (2022). Microbial and photodegradation of AFB1 in two contrasting soils (oral presentation). 43rd Mycotoxin Workshop, Toulouse, France.
- **Albert, J.**, More, C. A., Dahlke, N. R., Steinmetz, Z., Schaumann, G. E., & Muñoz, K. (2022). Validation of a Simple and Reliable Method for the Determination of Aflatoxins in Soil and Food Matrices (poster). 43rd Mycotoxin Workshop, Toulouse, France.
- **Albert, J.**, Steinmetz, Z., Fent, G., Schaumann, G.E. & Kubiak, R. (2019). Combined strategy to remediate a lindane polluted soil with the white rot fungus *Pleurotus ostreatus* and biochar (poster). SETAC GLB - Biodiversität im Wasser und am Land- Die Rolle chemischer Stressoren, Landau, Germany.

8.10 Electronic Supplement

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