

DECONTAMINATION OF MYCOTOXINS WITH ATMOSPHERIC PRESSURE PLASMA

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Doctoral Dissertation
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DEKONTAMINACIJA MIKOTOKSINOV Z UPORABO PLAZME
PRI ATMOSFERSKEM TLAKU

Doktorska disertacija

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*To my beloved Vito, family and friends.
To dear Mitja, who left us too soon.*

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Abstract

Mycotoxins are very stable toxic secondary metabolites produced by food contaminating filamentous fungal species. The variability of their chemical structure leads to a diversity of toxicities, including carcinogenicity, mutagenicity, teratogenicity, neurotoxicity and the immune and endocrine disruption. Today, 25% of the global cereal stock is contaminated with mycotoxins and unsuitable for consumption, which results in enormous economic losses. The incidence of mycotoxin food contamination is growing, and is problematic since it represents one of the main dietary risks. The increasing trend of mycotoxigenic fungi is mostly attributed to massive-scale food production, incorrect practices and handling of the food during harvest time, transport and storage, and climate change. Although the agriculture and food industry have explored a vast number of possible preventative and decontamination approaches, the problem of mycotoxin contamination remains unresolved. Thus, the development of a new and more effective method is necessary.

In this thesis, non-equilibrium cold atmospheric pressure plasma (CAP) is explored as a new promising approach for the decontamination of mycotoxins and inactivation of moulds. To achieve this, a highly efficient CAP system based on a surface barrier discharge (SBD) using ambient air as a carrier gas was applied for the decontamination of aflatoxins (AF), trichothecenes, fumonisins and zearalenone. CAP decontamination efficiency was compared to commonly used ultraviolet C (UV-C) irradiation and thermal treatment. Simultaneously, CAP mould inactivation potential was investigated with a focus on mould species *Aspergillus flavus*. Both direct exposure to the gaseous plasma and plasma-activated liquid broth (PAB) were compared to the conventional disinfectant Virkon®. The physical-chemical properties of CAP were evaluated, the degradation of aflatoxin B₁ (AFB₁) was studied in detail to determine the possible degradation products and pathways, and toxicity testing was performed.

It was demonstrated that CAP treatment of mycotoxins led to either their complete removal or reduction to a concentration below the maximum levels allowed in selected food products. The decontamination performance was found to be an order of magnitude higher in comparison to UV-C irradiation and thermal treatment. Moreover, the direct treatment with CAP successfully inactivated the spores of *A. flavus*. Under comparable experimental conditions, no inactivation was observed after the exposure of *A. flavus* to a standard 1% solution of Virkon®. However, PAB did not show the efficacy comparable to the direct treatment as a result of the intense hydrophobic properties of the surface of fungal spores. Analysis was performed on the CAP-generated reactive oxygen and nitrogen species (RONS) and it was determined that they contributed the most to its decontamination and inactivation effect, excluding thermal effect and plasma-induced release of UV photons. A detailed analysis of CAP RONS-induced degradation of AFB₁ demonstrated that the process was initiated through scission of the vinyl bond between C8 and C9 and continued through multiple pathways, resulting in the loss of the AFB₁ toxicity.

Lastly, the presented PhD thesis provides the proof-of-concept of CAP removal of mycotoxin contamination, which has the enormous potential for integration into already existing food production lines as a new highly-efficient and safe method for decontamination.

Povzetek

Mikotoksini so zelo stabilni toksični sekundarni metaboliti nitastih gliv, ki povzročajo onesnaženje živil. Zaradi zelo raznolike kemijske zgradbe različnih vrst mikotoksinov le-ti predstavljajo različne potencialne nevarnosti za človeka, saj so lahko rakotvorni, mutageni, teratogeni, nevrotoksični in povzročajo motnje imunskega in hormonskega sistema. Z mikotoksini naj bi bilo onesnaženih kar 25 % svetovnih zalog žita, ki so zato neprimerne za zaužitje. To posledično povzroča ogromne izgube v gospodarstvu. Pojavnost onesnaženja z mikotoksini nepretrgoma raste, zato predstavlja enega izmed najbolj zaskrbljujočih onesnaževalcev hrane. Prav tako narašča prisotnost mikotoksigenih plesni v hrani, kar je posledica vse večje proizvodnje hrane, nepravilnih praks pri ravnanju s hrano v času žetve, prevoza in skladiščenja ter celo podnebnih sprememb. Čeprav kmetijstvo in živilska industrija nudita številne preventivne in dekontaminacijske ukrepe, težave, povezane z mikotoksini, ostajajo nerešene. Zato je potreben razvoj nove metode za zmanjšanje onesnaženja hrane s pomočjo učinkovitejše dekontaminacije.

Predložena doktorska disertacija temelji na uporabi tehnologije neravnovesnih hladnih plazem pri atmosferskem tlaku (»cold atmospheric pressure plasma« – CAP), ki se je izkazala kot obetaven način za razgradnjo mikotoksinov in uničenje plesni. Plazemske obdelave v tej disertaciji temeljijo na uporabi površinske razelektritve (»surface barrier discharge« – SBD) zraka. Obdelave so potekale na različnih vrstah mikotoksinov, med drugim na aflatoksinih, trihoteceni, fumonizinih in zearalenonu. Učinkovitost dekontaminacije s plazmo smo primerjali z ultravijoličnim (UV-C) obsevanjem in toplotno obdelavo, torej metodama, ki se že rutinsko uporabljata v živilski industriji. Istočasno smo raziskovali tudi fungicidno aktivnost plazme. V ta namen smo plesen vrste *Aspergillus flavus* neposredno izpostavljali plinski fazi plazme in tudi plazemsko aktiviranemu tekočemu gojišču (»plasma-activated broth« – PAB). Učinkovitost smo primerjali s komercialnim razkužilnim sredstvom Virkon®. S pomočjo predhodno določenih fizikalno-kemijskih lastnosti in kemijske sestave CAP smo podrobno raziskali razgradno pot mikotoksinov na primeru aflatoksina B₁ (AFB₁), pri čemer smo natančno določili sestavo pomembnih produktov razgradnje ter testirali njihovo toksičnost.

Pokazali smo, da je obdelava s hladno plazmo pri atmosferskem tlaku bodisi popolnoma odstranila tretirane mikotoksine bodisi znižala njihove koncentracije pod dovoljeno mejno vrednost vsebnosti v izbranih živilih. Dekontaminacija s CAP je bila občutno bolj učinkovita v primerjavi z UV-C obsevanjem ali toplotno obdelavo. Prav tako je neposredna obdelava s CAP plinsko fazo uspešno inaktivirala spore *A. flavus* in povzročila zmanjšanje števila novo zraslih kolonij za ~ 5 log. Pri primerljivih eksperimentalnih pogojih obdelava s standardno uporabljeno 1 % raztopino Virkon® ni povzročila sprememb v številu novo zraslih kolonij plesni. V nasprotju z neposredno obdelavo plazemsko obdelano gojišče ni pokazalo izrazitih fungicidnih učinkov, kar je posledica hidrofobnih lastnosti površine zelo odpornih spor plesni. Podrobna analiza plazme je pokazala, da so za dekontaminacijske in inaktivacijske lastnosti hladne plazme pri atmosferskem tlaku najbolj zaslužene plazemsko proizvedene reaktivne kisikove in dušikove vrste (»reactive oxygen and nitrogen species« – RONS). Izkazalo se je tudi, da imajo plazemsko povzročeno povišanje temperature in plazemski UV fotoni pri tem zanemarljiv učinek. Reaktivne kisikove and dušikove vrste so tako povzročile razpad AFB₁, ki se je začel pri dvojni vezi med C8 in C9. Temu je sledila nadaljnja razgradnja po različnih poteh. Na podlagi tega je bila toksičnost AFB₁ odpravljena že po zelo kratkih časih obdelave.

Doktorska disertacija tako predstavlja preizkus koncepta novega pristopa, ki temelji na sistematično pridobljenih rezultatih dekontaminacije mikotoksinov z uporabo hladne plazme pri atmosferskem tlaku in na ta način metodo postavlja korak bližje k varni uporabi za industrijsko predelavo živil.

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Abbreviations

[M+H] ⁺	...	Protonated molecular ion
0D	...	Zero dimension
3-AcDON	...	3-Acetyl Deoxynivalenol
15-AcDON	...	15-Acetyl Deoxynivalenol
1D	...	One dimension
¹ H- ¹³ C HMBC	...	Heteronuclear Multiple Bond Correlation two-dimensional nuclear magnetic resonance spectroscopy
¹ H- ¹³ C HSQC	...	Heteronuclear Single Quantum Coherence two-dimensional nuclear magnetic resonance spectroscopy
¹ H- ¹ H TOCSY	...	Total Correlation two-dimensional nuclear magnetic resonance spectroscopy
¹ H-NMR	...	Proton Nuclear Magnetic Resonance
2D NMR	...	Two-Dimensional Nuclear Magnetic Resonance
AC	...	Alternating Current
AFB ₁	...	Aflatoxin B ₁
AFB ₁ -N ⁷ -Gua	...	<i>trans</i> -8,9-dihydro-8-(N ⁷ -guanyl)-9-hydroxyaflatoxin B ₁
AFB ₂	...	Aflatoxin B ₂
AFB _{2a}	...	Aflatoxin B _{2a}
AFG ₁	...	Aflatoxin G ₁
AFG ₂	...	Aflatoxin G ₂
AFM ₁	...	Aflatoxin M ₁
AFs	...	Aflatoxins
ALT	...	Altenuene
AME	...	Alternariol Monomethyl Ether
ANOVA	...	Analysis of variance
AOH	...	Alternariol
APPJ	...	Atmospheric Pressure Plasma Jet
Ar	...	Argon
ATR-FTIR	...	Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy
ATX I	...	Altartoxin I
ATX II	...	Altartoxin II
a-ZEN	...	a-Zearalenol
BDs	...	Barrier Discharges
b-ZEN	...	b-Zearalenol
CAP	...	Cold Atmospheric pressure Plasma
CD ₃ CN	...	d-acetonitrile
CFU	...	Colony-Forming Unit
DAS	...	Diacetoxyscirpenol
DBD	...	Dielectric Barrier Discharge
DC	...	Direct Current
diH ₂ O	...	Deionised water
DMSO	...	Dimethyl sulfoxide
DNA	...	Deoxyribonucleic acid
DON	...	Deoxynivalenol
e ⁻	...	Electron
EAs	...	Ergot Alkaloids
EDTA	...	Ethylenediaminetetraacetic acid
EFSA	...	European Food Safety Authority
EHD	...	Electrohydrodynamics
ESI	...	Electrospray Ionisation

EU	... European Union
FAO	... Food and Agriculture Organization
FAPY	... AFB1 formamidopyrimidine adduct
FB ₁	... Fumonisin B ₁
FB ₂	... Fumonisin B ₂
FBs	... Fumonisin
FDA	... Food and Drug Administration
FTIR	... Fourier-Transform Infrared Spectroscopy
H ⁺	... Hydrogen cation
H ⁻	... Hydrogen anion
H ₂ O ₂	... Hydrogen Peroxide
H ₃ O ⁺	... Hydronium
HCC	... Hepatocellular Carcinoma
He	... Helium
HepG2	... Human Hepatocellular Carcinoma cell line
HMDS	... Hexamethyldisilazane
HNO ₂	... Nitrous acid
HNO ₃	... Nitric acid
HO ₂	... Hydroperoxyl radical
HPLC	... High-Performance Liquid Chromatography
HRMS	... High Resolution Mass Spectrometry
IR	... Infrared
iso-TeA	... iso-Tenuazonic Acid
L-02	... Human embryo hepatocytes cell lines
LC-MS	... Liquid Chromatography coupled with Mass Spectrometry
LC-MS/MS	... Liquid Chromatography coupled with tandem mass spectrometry
LIF	... Laser-induced fluorescence
LMP	... Low Melting Point agarose
MRM	... Multiple Reaction Monitoring
MS	... Mass Spectrometry
MS/MS	... Tandem Mass Spectrometry
MTS	... (3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) salt
N	... Atomic nitrogen
N ₂	... Molecular nitrogen
N ₂ (A ₃ π)	... Electronically excited molecular nitrogen
N ₂ O	... Nitrous oxide
NaOH	... Sodium hydroxide
NIV	... Nivalenol
NMP	... Normal Melting Point Agarose
NMR	... Nuclear Magnetic Resonance
NO	... Nitric oxide
NO ₂	... Nitrogen dioxide
NO ₂ ⁻	... Nitrite
NO ₃	... Nitrate radical
NO ₃ ⁻	... Nitrate
N _x O _y	... Nitrogen oxides
O	... Atomic oxygen
O (1D)	... Electronically excited oxygen
O ⁺	... Oxygen cation
O ₂	... Molecular oxygen
O ₂ ⁻	... Superoxide
OES	... Optical Emission Spectroscopy
OH	... Hydroxyl radical
ONOO ⁻	... Peroxynitrite
OTA	... Ochratoxin A
OTs	... Ochratoxins
P	... Probability value or significance
PAB	... Plasma Activated Broth solution
PAT	... Patulin
PDA	... Potato Dextrose Agar
PMS	... Phenazine Methosulfate

PNNL	...	Pacific Northwest National Laboratory
RNA	...	Ribonucleic acid
RNS	...	Reactive Nitrogen Species
RONS	...	Reactive Oxygen and Nitrogen Species
ROS	...	Reactive Oxygen Species
SBD	...	Surface Barrier Discharge
SEM	...	Scanning Electron Microscopy
T	...	Temperature
TALIF	...	Two-photon Laser-Induced Fluorescence
TeA	...	Tenuazonic acid
TEN	...	Tentoxin
TMS	...	Tetramethylsilane
TSB	...	Tryptic Soy Broth
UPLC	...	Ultra-Performance Liquid Chromatography
UPLC-QqTOF MS	...	Ultra-Performance Liquid Chromatography system coupled with hybrid Quadrupole Time-of-Flight Mass Spectrometer
USA	...	United States of America
UV	...	Ultraviolet
UV-VIS	...	Ultraviolet-Visible spectrophotometry
WHO	...	World Health Organization
XTT	...	2,3-Bis-(2-methoxy 4-nitro-5-sulfophenyl)-2H-tetrazolium-5- carboxanilide salt
ZEN	...	Zearalenone

Chapter 1

Introduction

1.1 Atmospheric Pressure Plasma as a New Decontamination Tool

Plasma as the fourth state of matter is defined as a quasi-neutral ionised gas, which is composed of neutral and excited atoms and molecules, ions, free electrons and photons, as well as radicals and other reactive species [1]. Non-equilibrium cold atmospheric pressure plasmas (CAP) operate close to room temperature; hence, they can be used for the treatment of the sensitive matter, such as living tissues, cells and polymeric materials. Plasmas are also widely studied as a new tool for sterilisation of microbial pathogens and degradation of toxic agents from various surfaces, removal of pollutants in water, cancer treatment and many other domains in environmental, medical, biological, and nanotechnology areas [2, 3]. The universal applicability of CAP technology is supplemented by its low-cost and easy to use nature, which leaves a negligible burden on the environment. In light of this, the purpose of the research topic included in this thesis is to investigate the potential of CAP to decontaminate mycotoxins, which represent one of the most important global dietary risks.

This chapter, therefore, provides an insight into the fields of interest of the thesis, starting with the exploration of the problem of mycotoxin food contamination in Section 1.2, and introduction into non-equilibrium atmospheric pressure plasma fundamentals in Section 1.3. Following this, the motivation of the thesis is presented in Section 1.4 with the objectives and outline highlighted in Section 1.5.

1.2 The Problem of Food Contamination with Naturally Occurring Toxins

The environment consists of components such as air, water, soil and food, which strongly affect the quality of life and represent a point of potential contamination. Food is an essential source of energy and nutrition for all living organisms, including harmful microbial pathogens. Known to contaminate a variety of different food products regularly, they can lead to food spoilage and consequent foodborne diseases. Besides, food can also contain naturally occurring toxins, which refers to all toxic compounds produced by living organisms such as plants, animals, algae, microorganisms and fungi. Toxins have blighted mankind for centuries since spoiled and contaminated food has led to many accidental poisonings, many cases of which have resulted in death. On the contrary, many toxins are considered as beneficial medical compounds used for a variety of health conditions [4].

Today, the toxicity and the risks of naturally occurring toxins are well explored. However, the food and agriculture industries are still facing severe losses related to food contamination. One of the main sources of food spoilage are moulds, capable of producing mycotoxins with severe and harmful effects on the health of humans and animals [5-7]. Pathogenic filamentous species of fungi are responsible for significant yield losses of crops, which have a huge economic impact within the food industry and globalised supply chain of foods such as maize, wheat, nuts, barley, cottonseed and coffee. It is estimated that around 25 % of the global cereal stock is affected by mycotoxins and therefore unsuitable for consumption [8, 9]. There is no direct data on the annual economic burden resulting from mycotoxin contamination within the countries of the European Union (EU). Nevertheless, according to the study of Mitchell *et al.* [10], it was estimated that aflatoxin contamination in United States of America (USA) alone could cause losses in the maize industry ranging from 52 million to 1.7 billion US Dollars annually. Additionally, more than half of the world population is chronically exposed to the mycotoxin aflatoxin B₁ (AFB₁), a harmful group 1 carcinogen, which is considered as the most potent natural carcinogen. AFB₁ is

responsible for up to 155,000 cases of hepatocellular carcinoma (HCC) liver cancer, most of which will result in mortality within several months of diagnosis [11, 12].

The growing trend of mycotoxin contamination is assumed to be an unstoppable and unpredictable problem. Mostly, the mycotoxin occurrence may be attributed to improper harvest scheduling and post-harvest and storage handling of the food [13]. However, mycotoxins are often found on food where good agricultural, processing, storage and transport practices have been carried out. Moreover, recent studies have revealed that there is a correlation between the increased presence of mycotoxins and recent climate changes [14, 15]. Parameters including increased temperature, moisture levels and plant stress-related response stimulate fungal growth and the production of mycotoxins [16]. Moreover, climate change impacts the global economy, where food is transported long distances from the producer to the consumer. These issues include changes in local climate, transport and long storage times which all contribute to increased food contamination [17].

Strict legislation and regulatory guidelines to control the major classes of mycotoxins in food and feed were adopted by many countries worldwide, which was recognised and accepted by public health and governmental authorities, including European Food Safety Authority (EFSA), Food and Drug Administration (FDA), Food and Agriculture Organization (FAO) and World Health Organization (WHO) [18-20]. Slovenia as a member of the EU is managing the official control of mycotoxins in important food products since 2008 [21]. The agriculture and food industry apply several decontamination approaches that can typically be divided into physical, chemical and biological methods. The current trends in food processing demand the use of non-thermal techniques with minimal influence on the quality and organoleptic properties of the treated food. Consequently, methods such as ultraviolet (UV) and gamma irradiation, ozonation and pulsed light treatment have been explored. Nevertheless, presently available measures do not achieve satisfactory decontamination efficiencies meaning a vast amount of food that would be otherwise fit for consumption is being discarded annually [13].

1.1.1 Mycotoxins

Mycotoxins occupy 400 different variations in chemical structure. They are produced by filamentous fungi which belong to phylum *Ascomycota*. The major fungi causing frequent and problematic contamination of food and feeds with mycotoxins belong to fungal genera *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps* and *Alternaria*. The term mycotoxin is used exclusively for fungi related to food products and animal feed, excluding toxins produced by mushrooms [22].

Vertebrate animal species are the most vulnerable to intoxication by mycotoxins, which may enter the organism by ingestion, inhalation or skin contact. Once in the body, mycotoxins are responsible for later events of health complications known as mycotoxicosis. The outbreaks of such diseases strongly depend on the season of the year and the type of crops. Besides, mycotoxins can cause a broad range of toxicities, including carcinogenicity, mutagenicity, teratogenicity, neurotoxicity and endocrine and immune disruption [23]. Mycotoxins can appear in the food chain due to crop infection, either by being consumed directly by humans or through livestock feed appearing in meat, milk or eggs [24].

In respect to their similarity in chemical structure, mycotoxins are divided into several major classes. Aflatoxins (AFs) are deemed the most toxic members of mycotoxins. They include aflatoxin B₁, B₂, G₁ and G₂ (AFB₁, AFB₂, AFG₁ and AFG₂), regarding their fluorescence colour under ultraviolet light (B for blue and G for green) and their thin layer chromatography mobility. The main AFs producer is *Aspergillus spp.*, with *Aspergillus flavus* and *Aspergillus Parasiticus* as a prevailing mould species [25]. Normally, they contaminate food products like cereals, seeds, nuts and spices. The contamination can occur before harvest in the field or after the harvest when the food products are stored under fungal favourable conditions. Chemically they belong to the group of difuranocoumarins. AFB₁ is considered to be the most toxic aflatoxin. Once in the liver it is metabolized to AFB₁-8,9-*exo*-epoxide, which interacts with DNA, resulting in the development of cancer [26]. When metabolized in the liver via cytochrome P450-associated enzymes, AFB₁ can be transformed in monohydroxylated derivate known as aflatoxin M₁ (AFM₁). When AFB₁ contaminated food is ingested by mammals, AFM₁ is excreted mostly into the milk [27].

Trichothecenes are a large group of around 170 different metabolites. The most important members are deoxynivalenol (DON), nivalenol (NIV), toxin T-2, toxin HT-2 and diacetoxyscirpenol (DAS). Generally, they are produced by *Fusarium spp.*, *Myrothecium spp.*, *Phomopsis spp.*, *Stachybotrys spp.*, *Trichoderma spp.*, *Trichotecium spp.*, and *Verticimonosporium spp.* All of them are soil fungi and are important plant pathogens in warm

climates, mainly infesting the crops in the field [28]. All members of this mycotoxin group contain a tetracyclic-12,13-epoxy trichothene skeleton. Their toxic activity is based on their ability to bind to 60s ribosomal subunit, which inhibits the process of the initiation, elongation and termination during the protein synthesis. Trichothecenes were also one of the first compounds, which were proven to be involved in the inhibition of the peptidyl transferase activity. In addition, they can also inhibit DNA and RNA synthesis [29].

Fumonisin can be found in 28 different chemical forms, with B series (FBs) as the most important group. They are mostly produced by *Fusarium* species from *Liseola* section with the *Fusarium verticillioides* (syn. *Fusarium moniliforme*) and *Fusarium proliferatum* as main producers, which commonly contaminate maize and maize products, especially in warmer regions with relatively high humidity [30]. FB₁ is the prevalent fumonisin, chemically, recognized as 1,2,3-propanetricarboxylic acid, or as 1,10-[1-(12-amino-4,9,11-trihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediyl]ester. FBs are known to strongly inhibit the enzyme ceramide (CER) synthase, which is involved in catalysis of the acylation of sphinganine (Sa) and recycling of sphingosine (So). This leads to increased concentrations of intracellular Sa and other sphingoid bases that are known to induce the cytotoxic effects [31].

Ochratoxin A (OTA) is the prevalent ochratoxin (OT), mainly produced by mould species *Aspergillus ochraceus*, which contaminates a wide range of crops as well as fruits. Thus, OTA can be found in beverages such as beer and wine [32]. OTs are made of polyketide-derived dihydroisocoumarin moiety which is bound to L-β-phenylalanine via its 12-carboxyl group by an amide bond. Once ingested in the organism, OTA is mostly associated with nephrotoxic effects. Some studies suggest that OTA can produce oxidative stress and damage DNA. Moreover, electrophilic metabolites of OTA can bind to nucleotide bases, potentially resulting in mutagenicity and carcinogenicity [33].

Fusarium species are also known for the production of zearalenone (ZEN) and its two most important derivatives a-zearalenol (a-ZEN) and b-zearalenol (b-ZEN). ZEN is a 6-(10-Hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone. Given that its chemical structure holds a resemblance to the human sex hormone 17β-estradiol it is able to bind to estrogen receptors, resulting in reproduction problems in both humans and livestock [34].

Ergot alkaloids (EAs) are produced by *Claviceps spp.* which may be found in a variety of economically important grains. The main representatives of EAs are ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine and the corresponding “-inine” epimers. All of these compounds contain a tetracyclic ergoline ring, which belongs to the group of tryptophan-derived alkaloids. Generally, EAs are known to react with numerous neurotransmitter receptors, resulting in conditions like ischemia, decreased body gain and changes in the levels of some hormones. Unlike other mycotoxins, EAs do not produce carcinogenic effects, however some studies indicated they could work as cytostatic agents [35].

Due to the antimicrobial properties, patulin (PAT) was first used as nose and throat spray to treat the common cold and as an ointment to treat skin infections. As many later studies revealed its relation to toxic effects to animals and plants, it was removed from the sale and prohibited for use. The most problematic is the production of PAT by *Penicillium expansum* which contaminates apples and apple products. PAT is a toxic lactone with its chemical name 4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one. It is able to produce a variety of toxicities, including cytotoxicity, mutagenicity, inhibition of the enzymes and disruption of the immune system [36].

Altertoxins I and II (ATX-I and II), Alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid and iso-tenuazonic acid (TeA, iso-TeA), tentoxin (TEN), altenuene (ALT), and AAL-toxins are the members of the mycotoxin group altertoxins produced by the moulds from *Alternaria spp.* Most commonly, they contaminate food such as fruit and fruit derived products, cereals, vegetable oil and tomato-based products [37]. There are three different structural variations of altertoxins: the dibenzopyrone derivatives (AOH, AME, ALT), the perylene derivatives (ALT-X-I and II) and the tetramic acid derivatives (TeA and iso-TeA). At the moment, no regulation is prescribed to control altertoxins. However, there is a strong urgency to change this in the future, since many recent studies revealed that altertoxins are able to produce toxic effects, such as mutagenicity and teratogenicity [38].

1.3 Fundamentals of Cold Non-Equilibrium Atmospheric Pressure Plasma

Matter can occupy different states; a solid, liquid and gaseous state. The fourth state is plasma, which is the prevailing state of matter in the universe [39]. In principle, if a gas is heated or exposed to an electrical field, the free electrons are accelerated. These accelerated and energetic electrons can collide with gaseous molecules, resulting in the formation of other species, such as

ions. The collision of these electrons with the background neutral atoms and molecules results in the different processes including ionisation, dissociation, and excitation, which further lead to the production of other accelerated electrons and species. These processes induce the chain reaction of further collisions, and the mixture of molecules, neutral atoms, excited molecules, molecular ions, atomic ions, other excited particles, photons and electrons is formed, known as plasma [40].

From a technological point of view, plasmas are generally used for the treatment of material surfaces, including plasma sputtering, etching, implantation, thin film deposition, polymerisation, functionalisation, and many others. Such processes are carried out in low-pressure plasmas as well as at atmospheric pressure conditions [41]. Low-pressure plasmas have been widely used for applications such as material processing and are very important in the manufacturing of semiconductor devices. Their main advantages relate to their ability to generate high energetic plasma ions, which are uniformly distributed throughout the whole substrate area [40]. However, the vacuum systems and their maintenance are expensive, and the size of the treated object is limited to the size of the vacuum chamber. Moreover, the low-pressure conditions cannot be applied for the materials containing water which is a case for the majority of the biological samples [41].

The use of atmospheric pressure plasma technology avoids the disadvantages of vacuum systems. Atmospheric pressure plasma can consist of electrons and heavy particles (*i.e.* atoms, molecules and ions) with equivalent kinetic energies, meaning that they are in thermal equilibrium. Such plasmas are recognised as plasma arcs, which are typically very hot ($T > 5000$ K) [42]. At such temperatures, the thermal effect of plasma is comparable to its chemical effect. In contrast, the particles in plasma may also reach the state, where the electrons have considerable higher kinetic energies, whereas atoms, molecules and ions reach considerably lower temperatures. Such plasmas are called thermally non-equilibrium plasmas. The gas temperature of 'hot' non-equilibrium plasmas such as gliding arcs and plasma torches can rise to 4000 K, with electron energies only 10^4 K (1 eV). Highly non-equilibrium plasmas contain electrons with high kinetic energies, *i.e.* 10^5 K (10 eV). Due to their low gas temperature (300 – 400 K) they are also acknowledged as 'cold' atmospheric pressure plasmas (CAP), which opens up the possibility to use them for the treatment of the materials sensitive to heat. As such, they possess the potential to be used as a new tool in the field biology, medicine, environmental and food technology [43]. They appear to cause imperceptible damage to treated substrates, like cells and living animal or plant tissues, but on the other hand, they are capable of completely deactivating harmful agents like pathogen bacteria or toxic pollutants [3]. Given that their composition and temperature are adjustable, a variety of CAP sources have been designed and constructed, including the set-ups such as frequently used plasma jets and barrier discharges. Each type of source is capable of generating unique plasma chemistry. Short-lived species are the most important in the direct gaseous treatments, especially where treated surface acts as one of the electrodes, whereas the remote-indirect plasma treatment mostly depends on the generation of more neutral and long-lived reactive oxygen and nitrogen species (RONS) [44].

Atmospheric pressure plasma jets (APPJ) are typically composed of a gas nozzle, which contains one or two electrodes. After the ignition, plasma is generated inside the nozzle and transported to the outside environment. APPJs can vary in type of the carrier gas, applied electrical operating parameters, electrode configuration and design. They can also be used remotely, where plasma effluent in the nozzle mainly consists of reactive species. In contrast, expanding plasma in direct APPJ treatment encloses free and high energetic electrons. In such configurations, the treated substrate represents a second or third electrode [2].

Barrier discharges (BDs) are defined by at least one isolating layer in the discharge gap. One variation of the BD is a volume barrier discharge with two electrodes and at least one insulating layer forming the discharge gap. One of the electrodes can be a substrate, which is then directly exposed to plasma [3]. Another type of BD configuration is represented by the surface barrier discharge (SBD), where both of the electrodes are in contact with the insulating barrier. In this case, the treated subject is not a part of the configuration; thus, the SBD enables an indirect treatment of the substrate. SBD configurations have the potential for decontamination or sterilisation of large-area surfaces and are highly scalable [45]. With the generation of uniform concentrations of RONS throughout the entire treatment area, these systems are especially relevant for the decontamination and inactivation purposes in food processing [13]. SBD systems based on ambient air are also used in the experiments included in this thesis. For this reason, further subsections focus on the plasma chemistry produced by such discharges and their biological/degradation effects.

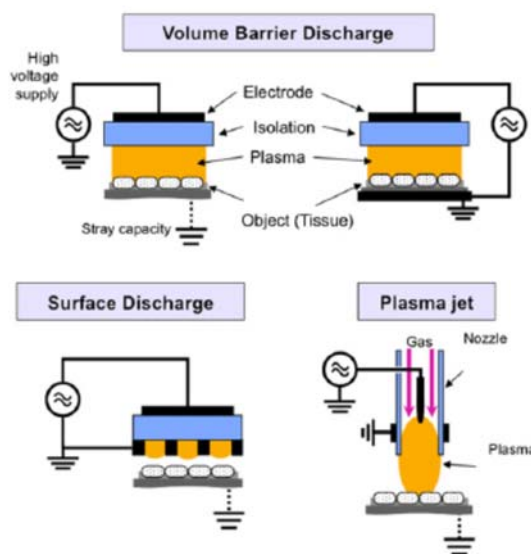


Figure 1.1: Highly non-equilibrium atmospheric pressure plasma sources suitable for biological applications [46].

1.1.2 The plasma-induced gaseous and liquid chemistry using air SBD plasma systems

The gaseous phase chemistry of plasma is initiated by applying an electrical field to the ambient air, which induces the acceleration of randomly appearing free electrons. These then collide with air molecules of oxygen (O_2) and nitrogen (N_2), inducing their ionisation, excitation and dissociation. Air molecules can also bind the seed electrons, resulting in the formation of negative ions. In addition, gaseous plasma contains photons, which are released from excited states of molecules and atoms. Consequently, many hundreds of the reactions in air occur, ending in the formation of ion fluxes, such as hydrogen ion (H^+), hydronium (H_3O^+), oxygen cation (O^+), hydrogen anion (H^-), oxygen anion (O^-), hydroxide (OH^-) and nitrogen cation (N_2^+); then molecular neutral species, such as N_2 , O_2 , ozone (O_3), and hydrogen peroxide (H_2O_2); and other reactive radicals, including atomic oxygen (O), hydroxyl radical (OH), nitric oxide (NO), nitrate radical (NO_3), collectively recognised as RONS [47].

The gas phase chemistry produced by an air SBD highly depends on the electrode geometry and applied voltage waveform. For this reason, it was a subject of various experimental and theoretical studies. Sakoyama *et al.* created a numerical model describing more than 50 species and 600 elementary reactions made by an atmospheric pressure surface microdischarge in humid air [47, 48]. The proposed model consisted of two zero-dimensional (0D) models, one in the area of discharge attaching the densities of short-lived charged and neutral species and second in the afterglow region following the long-lived neutral species. Furthermore, Liu *et al.* replaced 0D afterglow model with one dimensional (1D) model which describes the induced chemistry of deionised water placed downstream the electrode [49]. Most recently, Hasan and Walsh upgraded the two previous models into a single 1D reaction-diffusion model based on air SBD with the consideration of the convection on time scales much longer than the voltage waveform period, which takes into account the flow velocities around electrodes generated by the electrohydrodynamic (EHD) force of SBD plasma set-up. These velocities are believed to play a more significant role in species transport than diffusion. In short, Hasan and Walsh model grouped the plasma species into charged and neutral species [50].

Charged species such as N_2^+ and O_2^+ are generated and destroyed in one period of the applied waveform. With their short lifetime, they reach a maximum of 100 μm distance from the electrode. The spatial distribution of negative ions is wider compared to that of the positive ions as a result of electrons diffusing away from the dielectric to the regions where they are lost in attachment reactions. Neutral species consist of three subgroups: short-lived species, intermediate-lived species, and long-lived species. Short-lived species are in the majority electronically excited states such as $N_2(A^3\Pi)$ and $O(^1D)$ with a spatial distribution similar to electrons. Their lifetimes are shorter than a single period of the applied waveform. Intermediate-lived species, on the other hand, have the lifetime comparable to the duration of the applied

waveform. They accumulate over many periods, reaching over 100 μm from the electrode. They include species such as OH and N. The third category refers to long-lived species, which have lifetimes much longer than an applied voltage waveform period. Species such as O_3 , nitrogen oxides (N_xO_y), hydroxyperoxyl radical (HO_2) and H_2O_2 are widely distributed through the simulation domain even in the absence of convection and can be detected far from the dielectric surface [50]. To transform a model into more applicative form, the species can be generally characterised as short-lived (≤ 100 μm from the electrode) and long-lived species (> 100 μm from the electrode). Considering the lifetime of the RONS, the treated subject can be exposed to different plasma reactive chemistries, regarding the distance between the electrode and the position of the sample [7].

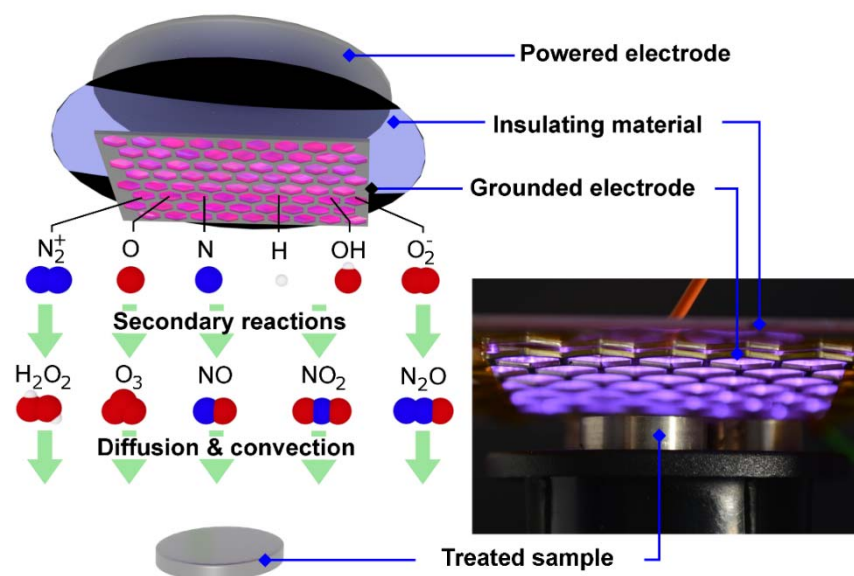


Figure 1.2: Surface Barrier Discharge (SBD) plasma based on the ambient air; a schematic of the plasma-induced gaseous chemistry.

The plasma effluent can also interact with a liquid interphase, resulting in unique chemistry, created through a wide range of physical-chemical processes and modifications [51]. Plasma-induced liquid (*i.e.* plasma “activated” liquid) modifications occur through two typical configurations; in the first scenario, the plasma phase is in the direct contact with the liquid volume, which enables the diffusion of the RONS as well as electrons and ions. Such plasma-liquid interactions are characteristic for plasma jets, where modifications are also promoted through photochemical reactions induced by plasma generated UV photons [52]. Plasma-induced chemical reactions in the liquid phase depend on various factors. Apart from the chemical composition of the gaseous plasma effluent, plasma-liquid modifications are strongly influenced by the initial liquid properties, especially its composition, which may contain electrolytes and radical quenchers. Plasma-liquid interaction can be affected by the conductivity properties of the treated liquids. During the treatment process, the liquid may also evaporate, which can reversely modify the chemistry of the gaseous plasma phase [52].

In the case of an air SBD plasma, the gap between the electrode and the liquid surface (> 100 μm) usually prevents the short-lived species from having a direct influence on the plasma-induced liquid chemistry. Therefore, the modifications in liquid are mostly conducted by long-lived RONS, especially O_3 , H_2O_2 , N_xO_y , HNO_2 and HNO_3 . The rate of transport of the gas-phase species into the liquid is determined by different factors, including the gaseous temperature and density and the pH value of the liquid. After the diffusion, the liquid volume based on deionised water mostly contains species such as H_2O_2 , NO_2^- , NO_3^- and H^+ . The latter induces a significant drop in pH, which influences further diffusion of gaseous RONS. Long-lived liquid species react with each other, resulting in the *in situ* generations of short lived-species such as peroxynitrite (ONOO^-) and OH [49, 53].

The chemistry in the gaseous plasma and liquid phase contribute the most to the antimicrobial properties of plasma. The mechanisms of plasma inactivation are supported by the presence of RONS such as OH, H_2O_2 , ONOO^- and O_3 , which interact with the cell membranes or walls. This

enables RONS to enter the cell and react with intercellular components like important organelles and DNA, resulting in the eventual cell death. Additionally, in plasma-treated liquids, the antimicrobial effect can also be achieved with the drop of the pH due to the significant concentrations of H^+ released from HNO_2 and HNO_3 . Moreover, RONS and the plasma-induced acidic conditions also influence the decay of complex molecules, such as various toxic compounds of natural or artificial origin. In brief, RONS usually attack the energetically less stable parts of such molecules, resulting in the complete degradation or transformation into less harmful compounds [13].

1.4 Motivation for the Use of Atmospheric Pressure Plasma for Mycotoxin Decontamination

Mycotoxins represent a complex problem for the modern food industry, a part of which are also inefficient decontamination methods that are currently used for food processing [13]. Recently, novel preliminary results based on food processing technology that involved the use of non-equilibrium gas plasma demonstrated enormous promise to reduce food contamination and improve food safety [54]. The key element of the decontamination approach based on CAP is its high chemical reactivity as a result of CAP generated RONS, which is achieved under nearly room temperature conditions. Consequently, the decontamination of toxic compounds and inactivation of the microorganisms is completed in short processing times. This is particularly interesting for thermally sensitive products such as the majority of foods. Moreover, CAP can be applied for the treatment of the pre-packaged foods to prolong the food product shelf life. So far, most of the research in this field was restricted to the removal of pathogenic bacteria. In very short exposure durations, lasting from a few seconds to 3 min, CAP treatments reached 5 log reduction for a wide range of bacteria species, including *Salmonella typhimurium*, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Campylobacter coli*, and even sporulating and toxin-producing species *Bacillus cereus* and *Clostridium botulinum* [55]. During recent years, different CAP systems were also proven to be effective for the inactivation of mycotoxigenic fungal species from genera *Aspergillus*, *Penicillium*, *Fusarium* and *Cladosporium* [56].

The reactive chemistry created by CAP-generated RONS was also used as a tool for the degradation of various toxic compounds such as soil and water pollutants. These include compounds such as atrazine, organophosphorous pesticides such as chlorfenvinfos, azo dyes, phenols, organochlorines and antibiotics [57-59]. Recently, much attention was dedicated to the CAP-mediated decontamination of mycotoxins. The first results based on the preliminary studies demonstrated that CAP treatment successfully removed different types of mycotoxins, including AFB₁, DON, ZEN, enniatins, FB₁, T2 toxin, sterigmatocystin and AAL toxin [13, 60-63]. Satisfactory decontamination rates were also achieved when CAP was used for the treatment of the foods contaminated with mycotoxins [64]. Additionally, when compared to other decontamination methods, a magnitude higher decontamination performance was achieved with CAP treatments [65].

Despite these promising results, these studies deliver insufficient insight into the CAP-induced degradation of mycotoxins, which prevents CAP technology from being further developed into a new processing method in the food industry. One of the main reasons for this lies within the EU regulation (EC) No 258/97 regarding novel food, which claims that any novel process used in the food production must not significantly alter the composition or structure of foods or food ingredients which affect their nutritional properties, metabolism or increase the level of undesirable substances. It is known that RONS are responsible for the high decontamination efficiency of CAP, yet, very little is known about the nature of these species, their interactions and effect on undesired contaminants, the degradation pathways and possible production of the toxic degradation products and the impact on the properties of the treated food products. To make progress in this area, a far greater level of understanding is essential.

The aim of this thesis is to overcome these barriers by the application of a systematic experimental approach to establish a complete picture of the complex interactions between CAP-generated RONS and molecules of mycotoxins. Thus, the focus is on the determination of the right treatment conditions required for the decontamination of the mycotoxins with an air SBD plasma system. With the emphasis on AFB₁ as the most toxic mycotoxin, this research includes uncovering the plasma-mediated degradation pathways, determination of the most important degradation products and evaluation of the possible toxic metabolite formed. In addition, the

fungicidal effect of CAP is investigated, where the treatments are carried out under CAP parameters comparable to those used for the decontamination of mycotoxins.

1.5 Thesis Objectives and Outline

The previous sections revealed the background of CAP technology, which exhibits a promise as a novel food processing method. The deactivation properties of CAP are mostly attributed to the unique plasma chemistry, which includes highly oxidative RONS. As indicated by some studies, RONS produced within CAP are highly effective antimicrobial agents that are capable of degradation of a variety of toxic compounds, including mycotoxins [13]. While the use of CAP as a decontamination tool in the food industry has immense potential, its uptake was restrained due to the lack of fundamental understanding of the complex interactions between CAP-generated RONS and biological materials.

This thesis considers these challenges, starting with the investigation of the relation between CAP operating parameters and the generation of RONS. This includes the carrier gas or CAP-treated liquid chemical composition, CAP electrode configuration and the electrical excitations, which all significantly affect the concentrations of CAP generated RONS and the ratio between reactive oxygen species (ROS) and reactive nitrogen species (RNS). Furthermore, the degradation products of mycotoxins as a result of CAP RONS are investigated as well as their potential toxicity. Exposure of mycotoxin molecules to plasma leads to changes in the chemical structure of the exposed molecules and creation of the new degradation compounds, which must be further precisely identified along with their toxic potential. The identification of major degradation products is essential for uncovering the degradation mechanisms, which are included in RONS decontamination of mycotoxins. Additionally, it must be considered that different plasma chemistries may induce the formation of different intermediate products and degradation pathways. Lastly, the optimisation of CAP treatment for mycotoxin removal has to be explored along with the fungicidal effects of the CAP RONS. In respect of this, the effects on fungi such as *A. flavus*, which is one of the main producers of the aflatoxins, have to be studied.

The thesis is therefore based on a systematic study, which includes a majority of the aspects required for the process to be accepted as a novel method for the purposes in the food industry. Moreover, the air SBD plasma configuration used for decontamination and inactivation is known for its high scalability. The low-cost and low-energy air SBD system is able to produce high concentrations of RONS, which are uniformly distributed over the electrode area. Moreover, the physical-chemical properties of such a system were a subject of several studies; thus the chemical processes are well defined.

Thesis objectives are:

- 1. To optimise the treatment process to obtain the most efficient CAP conditions for the decontamination of mycotoxins and inactivation of fungi.**

CAP decontamination and inactivation properties largely depend on the type of the low-temperature atmospheric pressure plasma system, known to produce different reactive chemistries. Plasma devices used in Chapters 2, 3, 4 and 5 consist of a high voltage power source operating at kHz frequencies and electrode unit, which is capable of generating several cm³ of gaseous air plasma on the dielectric surface. To identify ideal CAP decontamination conditions, different mycotoxins are exposed to plasmas created at different voltages and for different time durations (Chapters 2, 4 and 5). The decontamination efficiency is evaluated by the determination of the quantity of the remaining mycotoxin residuals. For this purpose, commercially available mycotoxins are sourced to provide a defined and pure chemical composition with precisely defined concentrations. The optimisation of the CAP inactivation of fungal spores is based on the experimental conditions comparable to those used for mycotoxin removal (Chapter 3). In addition, fungal spores are also exposed to plasma-treated liquid. The fungicidal effect is measured with standard microbiological methods, which includes the determination of the fungal spore survival and metabolic activity.

- 2. To characterise the plasma discharge chemistry and key RONS in the gaseous and liquid phase of discharge.**

Plasma chemistry created by an air SBD is extremely complex. Therefore, many different techniques are required to obtain comprehensive characterisation. The nature of the

chemistry present in the discharge of atmospheric pressure plasmas is highly unstable and collisional; therefore, applying the right and reliable plasma diagnostics approach is challenging. Hence, many of the widely used methods for low-pressure plasmas are unsuitable (*e. g.* Langmuir probe). Fortunately, recent advances in active atmospheric pressure plasma diagnostic techniques have enabled the determination of the key plasma species, including OH and NO. Such methods are Fourier transform infrared spectroscopy (FTIR), optical emission spectroscopy (OES), laser-induced fluorescence (LIF), two-photon absorption laser-induced fluorescence (TALIF), and others. The identification of plasma species diffused in the liquid is usually characterised with various colourimetric methods (UV-VIS spectroscopy), fluorescent spectroscopy, different types of chromatography, scavenger methods and many others.

The determination of the plasma chemistry is introduced in Chapters 2 and 4. Plasma diagnostics are performed with FTIR to identify the long-lived species, such as O₃, H₂O₂, N_xO_y, HNO₂ and HNO₃, arriving at the treated sample surface. OES is used to identify excited particles and the vibrational and rotational temperatures of the plasma particles to evaluate the neutral gas temperature. In Chapter 2, the plasma-induced liquid chemistry is researched by the employment of the pH measurements and different colourimetric/photospectrometric methods to determine the quantity of the species such as H₂O₂, NO₂⁻ and HNO₃. These techniques enable the chemical composition and key species generated in the liquids at specific CAP operating conditions to be identified.

3. To identify the most important degradation products generated after the exposure of mycotoxins to RONS and to reveal the underlying degradation mechanisms.

The study presented in Chapter 5 refers to a detailed analysis of the RONS-induced pathways based on a case study of CAP decontamination of AFB₁. For that, the chemical composition and structure must be identified, applying standard techniques in organic chemistry, which include high-resolution mass spectrometry (HRMS), nuclear magnetic resonance (NMR) and FTIR. Each of these methods provides a piece of complementary information about the important degradation products, directly involved in the process of decontamination.

Chapter 5 provides a comprehensive analysis performed with HRMS and NMR to identify the most important degradation products. Together with the outcomes of the RONS composition characteristic for the specific CAP treatment conditions, the most probable breakdown mechanisms are revealed.

4. To determine the potential toxicity of degradation products.

The toxic potential of the degradation products of CAP-treated AFB₁ is a critical factor that dictates the ultimate usability of the tested approach. The information about toxic effects is a core component of the chemical safety assessment data required by regulatory agencies for registration of acceptance for different products. The safety of the unknown compounds is usually explored through the examination of toxicity at different levels, which include tests for cytotoxicity, genotoxicity, mutagenicity, estrogenic/androgenic/thyroid activity, enzyme-inhibition activity and others.

Based on the AFB₁ carcinogenicity, the toxicity of the degradation products is explored in Chapter 5, with the focus on cytotoxic and genotoxic effects.

According to the motivation and the objectives of the experimental work, this thesis exposes the following hypotheses:

- Cold atmospheric pressure plasma technology is an efficient method for the decontamination of the mycotoxins with minimal effect on the treated food.
- Cold atmospheric pressure plasma induces the inactivation of mould species *A. flavus* as the main producer of AFB₁.
- Plasma chemistry promotes the degradation of AFB₁ into non-toxic degradation products with a different chemical structure compared to the parental AFB₁ molecule.

Chapter 2

Mycotoxin Decontamination of Food: Cold Atmospheric Pressure Plasma *Versus* “Classic” Decontamination

This chapter provides a critical review of the most important currently used methods for mycotoxin decontamination of food and feed. Despite a variety of options, they do not provide a satisfactory decontamination efficacy; thus, the food industry is losing the battle against persistent mycotoxins. CAP is presented as a promising non-thermal method, which overcomes the majority of the drawbacks of the so-called ‘classic’ food processing methods. Besides its high mycotoxin destruction efficiency, which was demonstrated in preliminary investigations, it also offers good prospects in terms of cost efficiency, ecological neutrality and a negligible effect on the quality of food products. In addition, this chapter provides an overview of the current trends in the field of food processing and highlights the state-of-the-art in CAP decontamination of mycotoxins.

This chapter addresses thesis Objectives 1.

Regarding my contribution: I performed the CAP treatment of the mycotoxins and UV-VIS spectrometry analysis and wrote the paper with co-authors.



Review

Mycotoxin Decontamination of Food: Cold Atmospheric Pressure Plasma versus “Classic” Decontamination

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Abstract: Mycotoxins are secondary metabolites produced by several filamentous fungi, which frequently contaminate our food, and can result in human diseases affecting vital systems such as the nervous and immune systems. They can also trigger various forms of cancer. Intensive food production is contributing to incorrect handling, transport and storage of the food, resulting in increased levels of mycotoxin contamination. Mycotoxins are structurally very diverse molecules necessitating versatile food decontamination approaches, which are grouped into physical, chemical and biological techniques. In this review, a new and promising approach involving the use of cold atmospheric pressure plasma is considered, which may overcome multiple weaknesses associated with the classical methods. In addition to its mycotoxin destruction efficiency, cold atmospheric pressure plasma is cost effective, ecologically neutral and has a negligible effect on the quality of food products following treatment in comparison to classical methods.

Keywords: cold atmospheric pressure plasma technology; mycotoxins; physical decontamination; chemical decontamination; biological decontamination

1. Introduction

Many species of filamentous fungi have the ability to produce toxic secondary metabolites known as mycotoxins. The term mycotoxin is used only for toxic substances produced by fungi related to food products and animal feed; it does not include toxins produced by mushrooms [1]. Today, about 400 structurally different mycotoxins have been discovered and divided into the following main groups: (i) aflatoxins produced by *Aspergillus* species and ochratoxins produced by both *Aspergillus* and *Penicillium* species; (ii) trichothecenes, zearalenone and fumonisins produced by *Fusarium* species; and (iii) ergot alkaloids, produced by *Claviceps* species, and others [2]. Generally, mycotoxins represent a significant threat to human health as they can be carcinogenic, neurotoxic and toxic to the endocrine or immune system [3]. They can appear in the food chain due to infected crops, which are either consumed directly by humans or used as livestock feed, appearing in meat, milk or eggs. Beside this,

they can contaminate food such as cereals, fruits, nuts, spices and other by-products as seen from Table 1 [4].

Table 1. Overview of the main characteristics of the most important mycotoxins.

Type	Representatives	Producing Fungi	Contaminated Foods	Structure Type	Toxicity
Aflatoxins (AF)	AFB ₁ , AFB ₂ , AFG ₁ , AFG ₂ , AFM ₁	<i>Aspergillus</i> spp.: <i>A. flavus</i> <i>A. parasiticus</i> <i>A. nomius</i> <i>A. bombycis</i> <i>A. pseudotamari</i> <i>A. ochraceus</i> <i>Aspergillus</i> spp. and <i>Penicillium</i> spp.: <i>A. ochraceus</i> <i>A. alliaceus</i> <i>A. auricomus</i> <i>A. carbonarius</i> <i>A. glaucus</i> <i>A. melale</i> <i>A. niger</i> <i>P. nordicum</i> <i>P. verrucosum</i>	Crops, cereals, seeds, nuts, spices	Difuranocoumarins	Carcinogenicity
Ochratoxins (OT)	OTA, OTB, OTC	<i>Fusarium</i> spp.: <i>F. verticillioides</i> <i>F. proliferatum</i> <i>F. napiforme</i> <i>F. dimerum</i> <i>F. nygamai</i> <i>Fusarium</i> spp.: <i>F. graminearum</i> <i>F. culmorum</i> <i>F. cerealis</i> <i>F. equiseti</i> <i>F. verticillioides</i> <i>F. incarnatum</i>	Crops, fruits, beer, wine, juices, coffee	Polyketide-derived dihydroisocoumarins bound to L-β-phenylalanine by amid bond	Nephrotoxicity, mutagenicity, carcinogenicity
Fumonisin	Series A (FA), B (FB), C (FC) and P (FP) with FB being the most common representatives: FB ₁ , FB ₂ , FB ₃	<i>Fusarium</i> spp.: <i>F. verticillioides</i> <i>F. proliferatum</i> <i>F. napiforme</i> <i>F. dimerum</i> <i>F. nygamai</i> <i>Fusarium</i> spp.: <i>F. graminearum</i> <i>F. culmorum</i> <i>F. cerealis</i> <i>F. equiseti</i> <i>F. verticillioides</i> <i>F. incarnatum</i>	Maize and its products	1, 2, 3-propanetricarboxylic acid	Cytotoxicity, carcinogenicity
Zearalenone (ZEN)	ZEN, α-zearalenol, β-zearalenol	<i>Fusarium</i> spp.: <i>F. graminearum</i> <i>F. culmorum</i> <i>F. cerealis</i> <i>F. equiseti</i> <i>F. verticillioides</i> <i>F. incarnatum</i>	Crops, cereals	6-(10-Hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone	Endocrine disruption
Trichothecenes	Deoxynivalenol (DON), nivalenol (NIV), T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS)	<i>Fusarium</i> spp. <i>Myrothecium</i> spp. <i>Phoma</i> spp. <i>Stachybotrys</i> spp. <i>Trichoderma</i> spp. <i>Trichotecium</i> spp. <i>Verticillium</i> spp.	Crops	Tetracyclic-12,13-epoxy trichothecenes	Inhibition of eucaryotic DNA, RNA and protein synthesis; nausea, vomiting, diarrhea, weight loss and loss of appetite, skin inflammation, vomiting, liver damage
Ergot alkaloids (EAs)	Ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine and the corresponding -inine epimers	<i>Claviceps</i> spp.: <i>C. purpurea</i>	Grains, grass	Tetracyclic ergolines (tryptophan-derived alkaloids)	Neurotoxicity, endocrine disruption
Other mycotoxins	Fusaproliferin (FUS), enniatins (ENN), beauvericin (BEA), moniliformin (MON), patulin (PAT)	<i>Fusarium</i> spp. <i>Penicillium</i> spp. <i>Aspergillus</i> spp. <i>Eupenicillium</i> spp. <i>Practolomyces</i> spp. <i>Byssodolomyces</i> spp.	Crops, fruits, vegetables, cereals	Sesterterpene cyclic hexadepsipeptides, 3-hydroxycyclobut-3-ene-1,2-dione, 4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one	Cytotoxicity, abnormal gluconeogenesis, genotoxicity and mutagenicity

Today, the trend of mycotoxins food contamination is increasing to alarming values with 25% of cereals worldwide already unsuitable for consumption [5]. Undesirable fungal growth and mycotoxin production is usually a result of incorrect agricultural and harvesting practices as well as the low effectiveness of prevention methods [3]. To reduce the potential danger to human health, many countries worldwide adopted strict legislation to control the mycotoxin presence in food and feed. In European Union, the presence of mycotoxins in food and feed is regulated by Regulation (EC) No 1831/2003, Directive 2002/32/EC, Recommendations 2006/576/EC and

2013/165/EU, and their amendments [6–9]. On top of this, recent studies have revealed a correlation between the increased presence of mycotoxins and global climate change [10]. Parameters including elevated temperatures, moisture levels and plant stress-related response stimulate fungal growth and, consequently, production of mycotoxins [10–13]. Furthermore, climate change plays a significant role in the global economy, where food is transported over long distances from producer to consumer, and may be subject to different local climates, transport and prolonged storage times. All these factors may contribute to increased food contamination [14].

Looking forward, it is expected that by the year 2050 the human population will exceed 9.2 billion. This will place an additional and unprecedented burden on the global food supply chain. The combination of modified climatic conditions and a tendency for consumers to eat healthier and fresher foods makes it imperative that new, sustainable and more effective approaches in agriculture, processing, transportation, and storage methods are developed. New mycotoxin-decontamination technologies will play a role in all stages of the supply chain. Beside this, the novel methods will have to preserve the quality of food products, be environmentally benign and economically suitable [14].

Considering the above-mentioned requirements, cold plasma technology represents a promising non-thermal mycotoxin-decontamination approach. Plasma is generally known as the fourth state of matter; a plasma state is reached by increasing the energy level of a substance from a solid state through the liquid and gaseous states of matter, ending in an ionized state of gas, which has unique physical and chemical properties (Figure 1) [15]. In electrically created plasmas, energy is delivered in the form of an electric field from an electrical power source; seed electrons produced by UV or background radiation are accelerated by the applied electric field leading to the excitation, dissociation or ionization of the background gas. Ionization, caused by the collision of an energetic electron with a neutral atom or molecule, results in the production of further electrons which are also accelerated in electric field. These free and energetic electrons subsequently collide with other surrounding molecules and atoms present in the gas, resulting in an avalanche process. Through the simultaneous generation and interaction among electrons, neutrals, metastables and ions, a vast number of reactions occur, yielding a wide variety of reactive chemical species [16,17]. In complex gas mixtures, such as humid air, a large number of reactive chemical species are created which take part in many hundreds of reactions [18]. In addition, molecules or atoms in an excited state can emit photons with wavelengths in the UVC, UVB and UVA range [19].

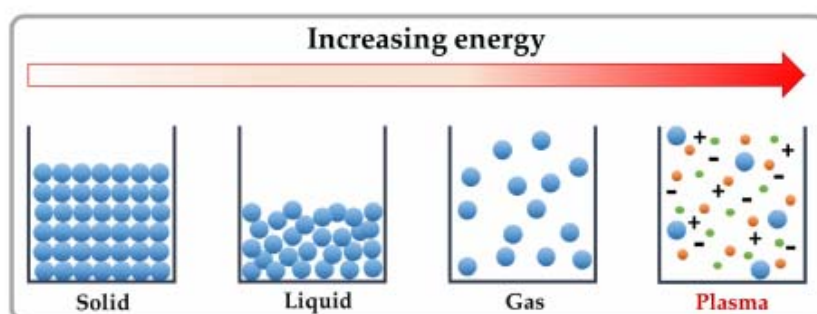


Figure 1. The generation of plasma: by adding energy to material, gas of electrons and ions is eventually produced. This fourth state of matter is referred to as “plasma”.

Plasma can be produced under low pressure or even atmospheric pressure conditions. Typically, low-pressure plasma systems require a discharge generator, a gas source and an expensive vacuum system, consisting of pumps and vacuum chamber. Such systems are widely used for applications in material processing. Nevertheless, they are not suitable for materials sensitive to low-pressure conditions including biological material [17]. The use of atmospheric pressure plasma avoids the disadvantages of vacuum systems and enables the treatment of biological materials. Common examples

of atmospheric pressure plasma systems include arc, corona and dielectric barrier discharges [20]. The most perspective discharges for the treatment of biological materials are those that are in thermal non-equilibrium, having a gas temperature that is close to room temperature, and are typically referred to as cold plasmas. Recent developments in cold atmospheric pressure plasma (CAP) sources and the ability to tailor discharges to produce highly reactive species in high concentrations, but at temperatures close to room temperature, have paved the way for a wide number of biological applications. Such CAPs are also suitable for use in electronics, surface modifications in polymer and textile industry, synthesis of nanoparticles and degradation of pollutants [21]. The new findings and developments in plasma science through the last decade reveal the great potential of CAP as an innovative technology in the field of biology, moving from the treatment of inanimate materials to living or cellular objects. Such applications include CAP treatments in medicine as well as in agriculture and the food industry. In the area of plasma medicine, research is mostly focused towards CAP use for skin treatment and wound healing, cancer cell and tumour treatment, dental implant sterilization, bone growth and many others [22].

CAP technology is, on the other hand, a newcomer to the field of agriculture and food industry. The main advantage of the CAP treatment of food products refers to its high chemical reactivity, achieved through the reactive species generated, and consequently its ability to deactivate harmful agents such as pathogenic bacteria and toxic pollutants in short processing times and at low temperatures with almost negligible impact on the treated food products. The technology can be applied to different types of food products in both solid and liquid form. In addition, the low energy consumption of such discharges and price-value inputs contribute to CAP being considered as an economically acceptable method [16,21,23,24]. Considering this, CAP technology also has a high potential as a decontamination tool for both mycotoxin-producing fungi and the mycotoxins that they produce. Different set-ups in both low and atmospheric pressure conditions have been used on mycotoxins such as aflatoxin B₁, deoxynivalenol and nivalenol (AFB₁, DON, and NIV, respectively) resulting in high decontamination rates in only a matter of seconds [25–27]. Treatments of the mycotoxin-producing fungi, e.g., one of the main mycotoxin producers *Aspergillus* spp. and *Penicillium* spp., with plasma, demonstrated very promising results as well. Plasma was able to stop or significantly reduce the further growth of fungi on different contaminated food products including corn, bean, cereals, fruits, nuts and many others [28–33]. Perhaps even more importantly, plasma treatments did not significantly influence the organoleptic characteristics of the treated foods or their nutritional properties [27,33–35]. Despite promising results, the use of CAP in the field of mycotoxin decontamination needs further exploration to uncover the CAP-related decontamination chemical processes and to develop optimised plasma systems suitable to meet the requirements of food processing and safety.

The aim of this contribution is to critically review plasma technology as a new food processing approach in the field of mycotoxin decontamination. The main advantages of this method over the classical mycotoxin-decontamination methods are considered and compared.

2. The Background of CAP Decontamination in Agriculture and Food Industry

A key benefit of the use of CAP technology in the field of agriculture and food is its high decontamination efficiency, which can be achieved in short treatment times and in non-thermal conditions. Its applicability has been widely demonstrated by successful decontamination of food with bacterial pathogens (*Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, etc.) and harmful compounds (phenolic compounds, pesticides, azo dyes, etc.) [21,35–43]. The mechanisms of CAP decontamination are attributed primarily to the highly reactive oxygen and nitrogen species (ROS and RNS) created within the plasma as well as UV radiation, which induce highly oxidizing effects [22,24,44–46].

The prevalent primary species in an air plasma include radicals such as OH•, H•, O•, and NO. These radicals can react with each other, and with the ambient/background gas (air), vapour or even liquids, where they create oxygen- and nitrogen-based secondary species such as H₂O₂, NO_x, O₃,

NO_2^- , NO_3^- , peroxyxynitrite, etc. These plasma species can be divided into short- and long-lived species depending on their lifetime [18]. Long-lived species can also exist after the plasma source is removed or turned off resulting in post-discharge reactions, which is named the plasma afterglow [44]. The importance of these species can be observed after primary interaction such as in the case of living cells, where these species first react with the cell plasma membrane, and later can enter the cell and cause damage to intercellular elements, such as organelles and biomolecules such as DNA, RNA and proteins. Similarly, when toxic compounds are exposed to the ROS and RNS produced in the plasma, they are decomposed directly or indirectly through secondary chemical processes with the transformation of toxic substances into less toxic reaction products (Figure 2) [47].

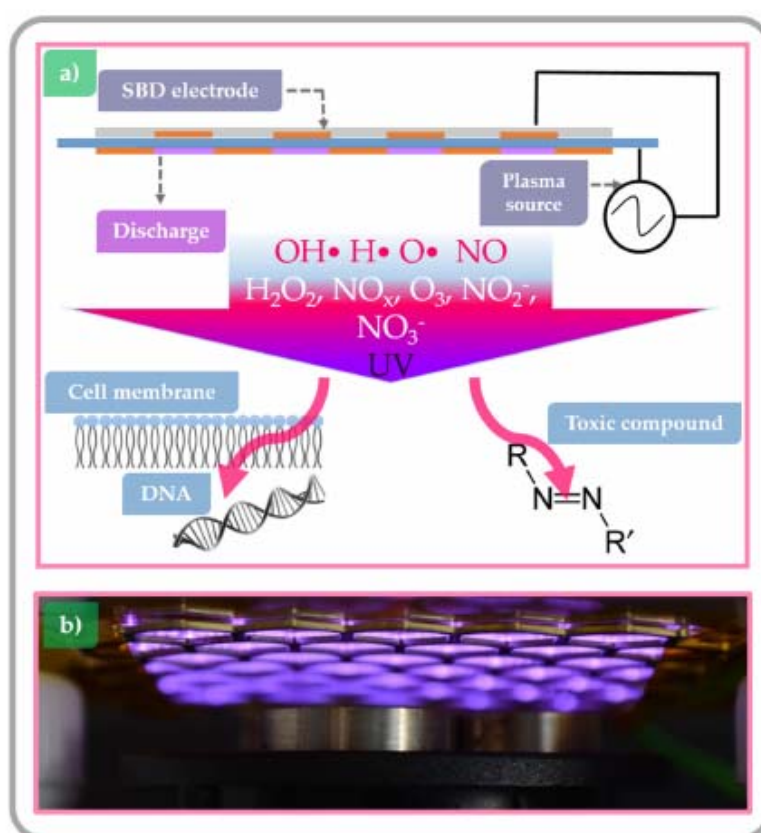


Figure 2. (a) Scheme of an air surface dielectric barrier (SDB) CAP set up; and (b) photo showing the CAP SDB system used in the presented experiments.

Among all plasma species, many studies have highlighted the key role played by atomic Oxygen (O), hydroxyl radical ($\text{OH}\bullet$), ozone (O_3), hydrogen peroxide (H_2O_2) and peroxyxynitrite in CAP-related decontamination effects, since they all possess a very high oxidative potential. In biological systems such as bacterial and fungal cells, the short-lived O and $\text{OH}\bullet$ first react with cell walls and membranes and with all the compounds composing these two structures (lipids, proteins and polysaccharides). The lipids are the most sensitive to oxidation. The mechanism of $\text{OH}\bullet$ reaction with lipids refers to its H-abstraction from the unsaturated carbon bonds of the fatty acids, ending in lipid peroxidation [44]. O_3 is also a powerful oxidant; ozonation alone represents one of the most potent sanitizing and detoxifying approaches in the food industry and mycotoxin decontamination. O_3 has high reactivity, penetrability, and spontaneous decomposition into non-toxic oxygen without forming harmful oxygen species. Compared to $\text{OH}\bullet$, O_3 induced reaction kinetics are slower [48]. In addition, the antimicrobial

activity of H_2O_2 is well explored. Generally, cytotoxicity caused by H_2O_2 begins with penetration into cells and then transformation to $\text{OH}\bullet$ through Fenton's reaction causing intercellular damage [49]. Peroxynitrite has recently been the object of many studies as it has been found to play an important role in oxidative stress and various diseases (neurodegenerative diseases, AIDS, arteriosclerosis, etc.) [50]. It oxidizes biomolecules directly or through H^+ - or CO_2 -catalysed homolysis. As for direct reactivity, it has affinity on key parts in proteins such as thiols, iron/sulphur centres, and zinc fingers. The lifetime of peroxynitrite is relatively short, nonetheless, it can still cross membranes and reach deep within the cell, which allows it to interact with most of the important biomolecules [51,52]. Regarding the CAP decontamination of toxic compounds, $\text{OH}\bullet$ as one of the strongest oxidative species initiates the toxic molecule oxidation, resulting in its degradation. However, other slower reaction pathways such as those caused by O_3 and H_2O_2 are shunted or even bypassed [43].

Plasma species production strongly depends on the CAP system design and its mode of operation. When building a plasma system for food processing, there is a wide range of operating or so-called discharge parameters to choose from, including different gasses (air, O_2 , N_2 , He, Ar, etc.) and gas flows, discharge types, discharge volumes, electrode setups, etc. The discharge can be generated using high-voltage electrical power sources or intense laser light [46]. In general, these systems can be divided into three groups defined by the position of the treated food product with respect to the point of plasma generation: at some significant distance from the generation point, relatively close to generation point or within the plasma discharge itself. With a change in position of the sample with respect to the plasma, the nature and flux of chemical species varies significantly and result in different surface effects [53]. The first category refers to remote treatment with CAP where the sample is physically separated from the plasma generation point. In this scenario, the plasma generated species are usually transported to the sample by diffusion or by an induced flow. By the time plasma species reach the targeted surface, they are mostly composed of longer-lived plasma species, with a negligible concentration of highly reactive species (Figure 3a) [54]. The second category of system enables a semi-direct treatment with CAP. Then the target is exposed to higher concentrations of short-lived and highly reactive chemical species due to the relatively short distance between plasma generation point and substrate. In this scenario, the flux of UV photons reaching the targeted surface is also relatively high (Figure 3b) [55]. The last category is known as a direct contact system where the sample is placed between the electrodes of the plasma generation system and is consequently bombarded by large fluxes of reactive species and UV light (Figure 3c) [56,57]. While direct treatment should offer the highest possible degradation and decontamination efficacy, its implementation is problematic. The sample forms part of the electrical circuit and its presence can disrupt the discharge leading to the formation of hot spots that can damage the product.

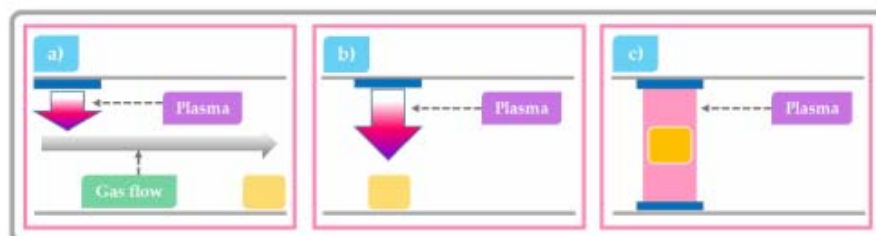


Figure 3. Schematic overview of common CAP systems considered for use in the food industry: (a) remote treatment where the sample is physically separated from the plasma generation point (b) semi-direct exposure, where the sample is placed close to the plasma generating electrodes; and (c) direct-exposure, where the sample is positioned between the plasma generating electrodes.

A wide range of design elements and discharge parameters enable a high degree of flexibility when designing CAP systems for food processing purposes no matter the type, size, and shape

of the treated food products [58]. In terms of mycotoxin removal, plasma technology has mostly been used for the treatment of seeds, cereals, crops and fresh products [30,34,58,59]. For example, the use of an atmospheric pressure fluidized bed plasma system with air and nitrogen as a feed gas, which was used for the inactivation of *A. flavus* and *A. parasiticus* contaminated maize resulting in a 5.48 log reduction [28]. Furthermore, the production of fumonisin B₂ and ochratoxin A (FB₂ and OTA, respectively) was inhibited after the exposure of *A. niger* on date palm fruits to an argon CAP source. Oxygen CAP was applied for the treatment of *C. cladosporioides* and *P. citrinum* on the surface of dried filefish fillets reducing the fungi by more than 90% [33]. Moreover, argon and oxygen CAP proved to be efficient against *A. brasiliensis* contaminating pistachios [60]. Siciliano et al. performed CAP treatment with different mixtures of oxygen and nitrogen for decontamination of AFB₁ from dehulled hazelnuts succeeding 70% decontamination rate [27]. One of the most interesting CAP applications is also the in-package treatment of food. In this scenario, strawberries were treated with CAP generated between the electrode gap and inside a sealed package. The background microflora containing fungal species was reduced by 2 log reduction which could significantly prolong the food product expiry date [35].

3. The Comparison of “Classic” Approaches and CAP Technology in the Field of Mycotoxin Decontamination

Actions for preventing the fungal and mycotoxin contamination of feedstuff are performed at critical points before the expected fungal infestation. This may occur at the pre-harvest stage, during the harvest-time or at the post-harvest handling and storage stages [61]. The most effective approach is primary prevention, which should be carried out before the fungal invasion and mycotoxin production occurs. Current approaches include the use of fungicides to inhibit fungal growth, an appropriate scheduling of harvesting, and maintaining the optimum storage conditions after harvest [62,63]. Unfortunately, such techniques are not entirely effective and the efficiency of fungicides varies for different fungal species [64]. For this reason, several recent approaches have focused on the development of fungi-resistant plants [65]. The various physical, chemical and biological methods for the reduction of mycotoxin contamination currently in use or under active investigation for food and feed products are reviewed below, and compared with CAP technology (Figure 4) [61].

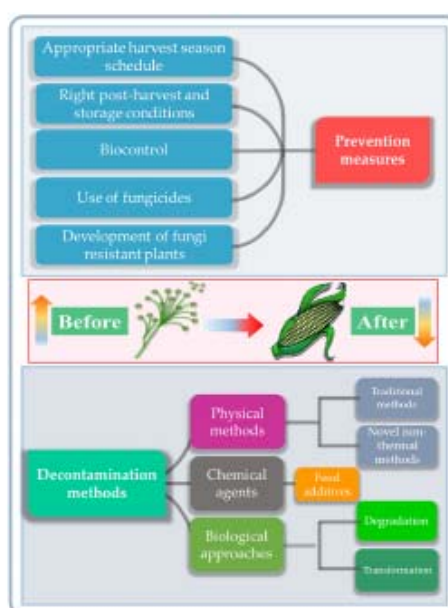


Figure 4. Overview of the currently available mycotoxin prevention and decontamination measures taken before and after fungal and mycotoxin contamination of food.

Methods for the physical decontamination of contaminated food are typically divided into traditional measures and novel non-thermal methods. The first group refers to methods that include sorting, washing, dehulling, density segregation, grain milling and thermal treatment. The principle of their decontamination is mostly based on the removal of the contaminated food parts and consequently the mycotoxins [66]. On the contrary, thermal treatment causes thermal degradation of mycotoxins [67–69]. Most of the traditional methods can reach satisfying decontamination rates for different types of toxins [2,70]. However, the processing time is usually very long, requiring high energy input, and is therefore very expensive. In addition, heat treatment can significantly affect the quality of the treated food products [61]. For these reasons, the food industry is looking to further develop new non-thermal approaches such as UV- and gamma-irradiation, pulsed-light treatment as well as CAP technology. Non-thermal methods typically affect the chemical structure of the mycotoxins leading to their degradation. Their decontamination efficiency depends on the presence of water in the treated food products, the extent of mycotoxin contamination, and the intensity of exposure [66]. For gamma irradiation technology, it has been reported that the decontamination of various mycotoxins was significantly more successful when they were present in solution, reaching up to 90% removal rate. The dosages of gamma irradiation used were from 1 to 20 kGy. Degradation in this case was probably a result of the formation of free radicals which were produced by the radiolysis of water. On the contrary, gamma irradiation decontamination of the mycotoxin-contaminated solids and dry food products in conditions with low moisture values was notably less effective [71]. High doses of gamma irradiation could, however, negatively affect the quality of food products such as grains and seeds, reducing their germination ability for example [72]. UV light irradiation also demonstrated high efficiency in mycotoxin decontamination. Treatments using a wavelength of 365 nm were capable of reducing the content of aflatoxins (AFs) from various types of nuts by more than 90% [73]. The 365 nm light irradiation was capable of removing the AFB₁ in peanut oil almost completely. Moreover, toxicity tests employing human embryo hepatocytes showed a significant reduction of toxicity of the degradation products [74]. When an AFB₁ aqueous solution underwent the UV light irradiation treatment, three major degradation products were observed (Table 2). It was indicated that the UV light irradiation probably interacted with most of the active sites on AFB₁, i.e., C₈-C₉ and O₁-C₁₄ bonds. These two bonds have been recognized as being responsible for the AFB₁ toxicity and were transformed to more stable saturated bonds [75]. Interestingly, the structure of AFB₁ degradation products depended significantly on the media. Different degradation products were identified when the treatment was performed in acetonitrile or in peanut oil solution (Table 2) [74,76]. UV light irradiation was also used for patulin (PAT)-contaminated apple juice. Performed at 222 nm, a 90% reduction of mycotoxin content was achieved. However, such treatments lowered the concentration of some other photosensitive substances in the juice, including healthy ascorbic acid [77]. Similar to UV light irradiation, Moreau et al. studied mycotoxin, OTA, ZEN, DON or AFB₁, decontamination with pulsed light. The light flashes used were 300 µs in duration with a broad spectrum of light ranging from 180 to 1100 nm and a light flux 1 J/cm². The analysis demonstrated that eight light flashes almost completely removed mycotoxins from the solution. Remaining toxicity was assessed on nematode *Caenorhabditis elegans*. Degradation products of DON and ZEN were evaluated as not toxic. The mutagenic activity based on an Ames test showed that AFB₁ degradation products were not mutagenic [78]. In a recent study, a pulsed light system of 0.52 J/cm²/pulse and 360 µs long flashes, with wavelengths ranging from 100 to 1100 nm were used to decontaminate AFB₁ and aflatoxin B₂ (AFB₂) on different rice products. Decontamination efficiency higher than 90% was achieved for AFB₁ after 15 s of treatment in the case of rice bran [79].

An alternate detoxification strategy employs chemical agents, which are able to detoxify mycotoxins when added to a contaminated feedstuff. The effect is achieved by many synthetic and naturally occurring compounds including various organic acids, ammonium hydroxide, calcium hydroxide mono-methylamine, hydrochloric acid, hydrogen peroxide, bisulphite, chlorinating agents, formaldehyde, ammonia, clove oil and many more [63]. Ammoniation is conventionally used for AFs

decontamination of feed such as cottonseed and peanut meal. The effectiveness of detoxification with ammonia increases with the quantity of ammonia used, the time of treatment, the temperature and pressure level [80]. Many types of ammoniation are available, with the two most commonly used being high-pressure/high-temperature treatment and atmospheric pressure/moderate temperature treatment. Both methods are able to reduce mycotoxin content up to 90%. For example, the degradation of AFB₁ by ammoniation is accomplished by hydrolysis of the lactone ring, which is followed by decarboxylation to AFD₁ and subsequent loss of cyclopentane ring (Table 2) [81–83]. Ozonation is a rather new way of mycotoxin decontamination in food processing [48]. When AF-contaminated corn flour was exposed to 75 mg/L of ozone for 60 min, the content of AFB₁, aflatoxin G₁ (AFG₁) and AFB₂ decreased from 53.60, 12.08 and 2.42 µg/kg to 11.38, 3.37 and 0.71 µg/kg, respectively [84]. In another study, 89.4% decomposition of AFB₁ was achieved after AFB₁-contaminated peanuts exposure to ozone of 50 mg/L at a flow rate of 5 L/min for 60 min. Following this results, the two most probable AFB₁-degradation pathways were proposed. In the first, the ozone initially reacts with a C₈–C₉ double bond of the furan ring in AFB₁ in electrophilic reaction based on Criegee mechanism, whereas the second degradation pathway starts with oxidation of the AFB₁ benzene methoxy group. Both reaction pathways lead to five final degradation products (Table 2). Generally, the toxicity of most degradation products is reduced under the assumption that a C₈–C₉ double bond represents one of the sites responsible for toxicity of AFB₁ [85]. Wang et al. used ozone to achieve a reduction in toxicity of DON contaminating wheat grains. After 60 min of 100 mg/L ozone treatment, the concentration of DON decreased from 3.89 mg/kg to 0.83 mg/kg, which is under the generally recognized maximum mycotoxin limit in feed [86]. Another method of chemical mycotoxin decontamination is the use of feed additives. These inorganic and organic mycotoxin binders are added to a feedstock when there is an indication of mycotoxin contamination. Typical additives include clays as natural mycotoxin adsorbents made of silicates or aluminosilicates. The level of adsorption of mycotoxins depends on the size and the charge of the mycotoxin with regard to the specific structure of the clay used [61]. The majority of clays are able to bind AFs, but not ZEN, fumonisins and trichothecenes, when added at a concentration of 10 g/kg [87,88]. On the other hand, bentonite was able to adsorb T-2 toxin, but to achieve high binding efficiency much more than 10 g of adsorbent per kg had to be used [89]. Inconveniently, clays are also able to adsorb the micronutrients from feed and disturb the bioavailability of minerals and trace elements. Furthermore, the contamination of clays with dioxins is possible [90]. As the inorganic adsorbents proved to be inefficient removers of the majority of mycotoxins, natural eco-friendly organic binders have been introduced instead, including oat fibres and cell extracts of lactic acid bacteria and *Saccharomyces cerevisiae* [91,92]. The decontamination of food by chemical means may be inexpensive and can achieve good decontamination results; however, most of these methods can present a risk for the environment as well as for human health. A further disadvantage is the long treatment time, which is not good for preservation of high quality foods.

The final category of mycotoxin decontamination measures includes biological methods. These procedures are based on the ability of microorganisms such as bacteria, yeast, moulds, actinomycetes and algae to remove or degrade mycotoxins in food and feed products. A clear advantage of biological decontamination approaches is that no chemicals are involved. The methods are based on biological transformation, enzymatic degradation, or modification of mycotoxins to less toxic substances. Mycotoxins can be thus acetylated, glucosylated, cleaved at their rings, hydrolysed, deaminated or decarboxylated [93]. Microorganisms capable of mycotoxin detoxification include species such as *Bacillus* spp., *Brevibacterium* spp., *Pseudomonas* spp., *Rhodococcus erythropolis*, *Aspergillus* spp., *Rhizopus* spp. and *Trichosporon mycotoxinivorans*. They can efficiently detoxify a wide range of mycotoxins including AFB₁, AFG₁, OTA, ZEN, PAT and DON [94–103]. In addition to reducing bioavailability of mycotoxins, some microorganisms, e.g., probiotic bacteria, are frequently added to feed as an additive with positive effect on the gut flora. The effectiveness of such microorganisms to act anti-mycotoxically largely depends on their ability to remain stable in the gastrointestinal tract. The main representatives of this group of bacteria are lactic acid bacteria [104]. Generally, biological approaches are not expensive

and their environmental impact is low. Nevertheless, mycotoxin decontamination processes using microorganisms can be quite time-consuming [93].

In comparison with the methods described previously, CAP mycotoxin decontamination of food overcomes many of the disadvantages and obstacles of physical, chemical and microbial decontamination procedures. As depicted in Table 3, most of the CAP systems used for decontamination of food are environmentally benign, require a low energy input and are economically favourable. Beside this, plasma approaches have proven to have a negligible effect on the quality of many types of treated food. These advantages are based on the reactivity of the plasma species which enable the high decontamination efficiency in a very short time compared to alternative decontamination methods [46,53,105]. To demonstrate the efficiency of the plasma approach, a microwave-induced atmospheric pressure plasma system was used with argon as a carrier gas to treat three different mycotoxins, AFB₁, DON, and NIV dried on glass coverslips. The treatment resulted in the complete decontamination of all three mycotoxins after only 5 s of plasma exposure. Plasma treatment completely eliminated their cytotoxicity as tested on mouse macrophage RAW264.7 cells in vitro [25]. Furthermore, low-temperature radiofrequency plasma was used to degrade AFB₁. After 10 min of treatment, 88.3% AFB₁ was degraded. Analysis of the degradation products indicated that the toxicity should be reduced based on the structure-activity criteria; the degradation pathways indicated the formation of five different decay products (Table 2), where plasma induced the loss of the double bond in the terminal furan ring (C₈-C₉) [26]. AFs were exposed to a dielectric barrier discharge (DBD) plasma system, resulting in the complete destruction of mycotoxins when they were treated alone. Using the same plasma system, a 70% decontamination level was achieved for the treatment of AFB₁ contaminated dehulled hazelnuts [27]. To demonstrate the effectiveness of CAP, our recent experiments consider the use of an air surface barrier discharge (SBD) plasma treatment compared with UV light irradiation or thermal treatment in regards to AFB₁-destruction efficiency. Standard solution of AFB₁ was prepared in the mixture of acetonitrile and deionized water (2:1). One hundred microlitres of AFB₁ standard solution was applied on the glass coverslips and dried for 5 min. Such wet samples were then exposed to CAP, UVC light, or thermal treatment. CAP set-up was similar to the one reported by Ni et al. [40] and was operated at three different discharge powers (P_d): low (10 W), medium (15 W) and high (20 W). Low P_d operated plasma mostly contained ROS whereas RNS were the prevalent species at high P_d conditions. Plasma was observed to achieve more than 80% destruction level after just 15 s of treatment of AFB₁ applied on, regardless of the P_d used. In contrast, no significant transformation of AFB₁ was observed under thermal or UV light treatments, even at the longest exposure times (Figure 5). The ability of plasma to rapidly affect the AFB₁ molecular structure was confirmed by UV-Vis spectrometry. As evident from Figure 6, both major peaks in the UV-Vis spectra of AFB₁ significantly changed after 8 min of exposure of AFB₁ to plasma, independently of the P_d . On the other hand, the AFB₁ UV-Vis spectra remained almost the same following the UV or thermal treatment for the same time period. UV light irradiation treatment is usually efficient in degrading only the mycotoxin molecules, in particular AFs, which are known for their photosensitivity [74–76]. Beside this, UV irradiation represents one of the most commonly used decontamination approaches in food processing [106]. Comparing to CAP, to achieve adequate results, this method requires much longer exposure times (more than 10 min compared to some seconds in the case of CAP). Here, it is worth mentioning that UV requires higher power inputs which further impacts the decontamination efficiency. In addition to the mentioned drawbacks, it has been reported that UV irradiation could even increase the mutagenicity of AFs [107]. The characteristics of mycotoxin plasma treatment can be compared to some extent with ozone treatment, since one of the prevalent plasma-produced long-lived molecular species is ozone [108]. As many other reactive species beside ozone are produced in the plasma, synergistic effects can occur, resulting in the mycotoxin decontamination of food requiring significantly less exposure times than ozone alone [48,84,85]. Despite numerous advantages, CAP technology also has some limitations. One of the major problems is an inability to precisely control the gas phase chemistry when using ambient air, given that it varies with conditions in the surrounding atmosphere

(for example increases in humidity). Since CAP contains ROS, it is not suitable for the treatment of high-fat food products. Furthermore, when carried out using very high voltages, additional safety measures are required as well as systems for the destruction and exhaust of potentially harmful long-lived species such as O_3 and NO_2 [46].

Table 2. Degradation products of AFB₁ after treatment with different decontamination methods.

Decontamination Method	Degradation Products	Reference
	In aqueous solution:	
	AFB₁ C₁₇H₁₂O₆ MW=312 P₁ C₁₇H₁₄O₆ MW=314 P₂ C₁₇H₁₄O₆ MW=284 P₃ C₁₄H₁₀O₆ MW=274	[75]
UV	In acetonitrile:	
	AFB₁ C₁₇H₁₂O₆ MW=312 P₁ C₁₇H₁₄O₆ MW=330 P₂ C₁₇H₁₄O₆ MW=302 P₃ C₁₇H₁₄O₆ MW=316	[76]
	In peanut oil:	
	AFB₁ C₁₇H₁₂O₆ MW=312 P₁ C₁₇H₁₄N₂O₆ MW=339 P₂ C₁₇H₁₄N₂O₆ MW=226	[74]
Plasma	AFB₁ C₁₇H₁₂O₆ MW=312 P₁ C₁₇H₁₄O₆ MW=222 P₂ C₁₆H₁₇O₆ MW=353	[26]

Table 2. Cont.

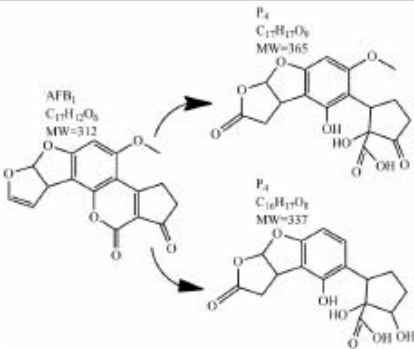
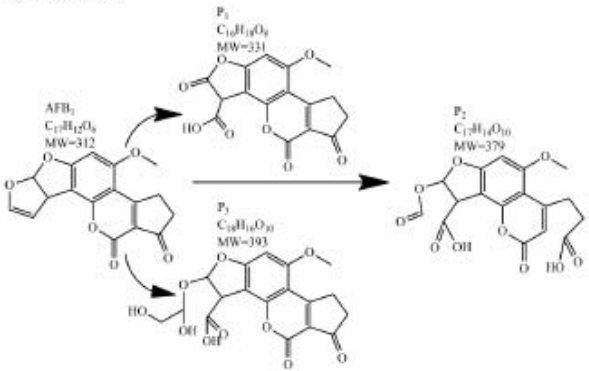
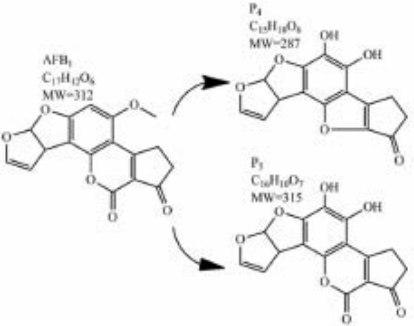
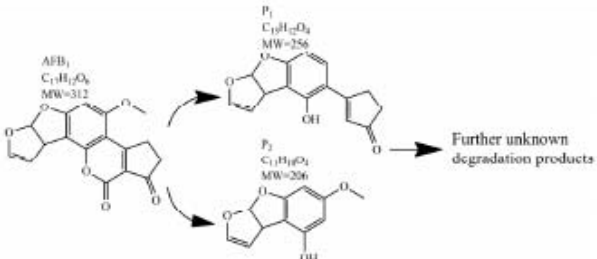
Decontamination Method	Degradation Products	Reference
		
	In acetonitrile:	
		
Ozone		[85]
Ammoniation		[81]

Table 2. Cont.

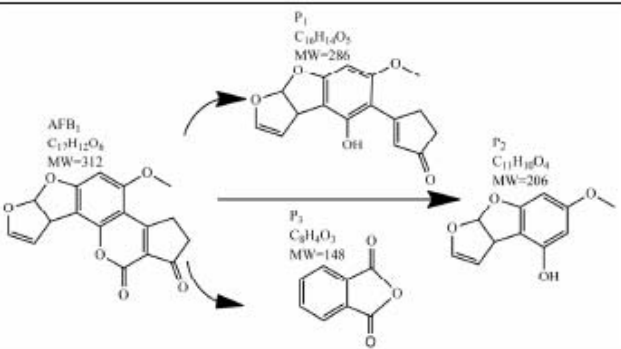
Decontamination Method	Degradation Products	Reference
<i>P. putida</i>		[96]

Table 3. The comparison between mycotoxin decontamination methods.

Decontamination Method	Highest Decontamination Rate Obtained	Food Product	Process Duration	Energy Consumption	Impact on the Food Quality	Reference
Thermal treatment	85–100% (FBs, ZEN, AFs)	Corn	Long	High	Significant	[70,109,110]
Gamma irradiation	90% (mixture)	Grains, seeds	Short	Low	Significant	[71]
UV light irradiation	90% (AFB ₁ , PAT)	Peanut oil; apple juice;	Short	Low	Negligible	[74,77]
Pulsed light technology	90% (AFB ₁)	Rice products	Short	Low	Negligible	[79]
Ammoniation	90–100% (AFB ₁)	Rice	Long	High	Significant	[83]
Ozonation	80% (AFs)	Corn flour, peanuts	Long	Low	Negligible	[84,85]
<i>Bacillus</i> spp.	92.5% (OTA)	/	Long	Low	Negligible-significant	[111]
<i>Rhodococcus erythropolis</i>	90% (AFB ₁)	/	Long	Low	Negligible	[98]
<i>Aspergillus</i> spp.	100% (ZEN)	/	Long	Low	Negligible-significant	[99]
<i>Trichosporon mycotoxinivorans</i>	100% (OTA)	Animal Feed	Long	Low	Negligible	[103]
Lactic acid bacteria	80–100% (FBs)	/	Long	Low	Negligible	[112]
CAP technology	100% (AFs, DON, NIV)	Seeds, crops, cereals	Short	Low	Negligible	[25,27]

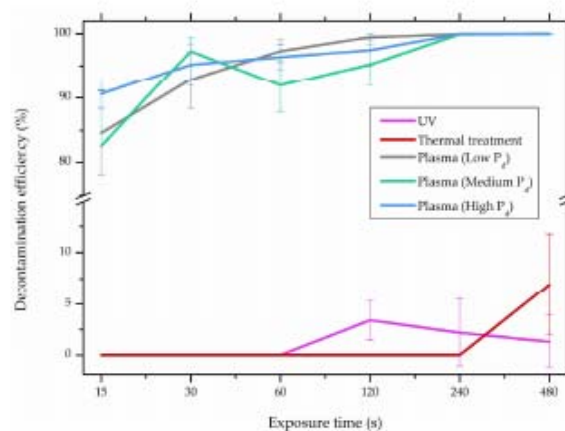


Figure 5. Comparison of decontamination efficiency (%) of aflatoxin B₁ (AFB₁) between cold atmospheric pressure plasma (CAP) and conventional decontamination approaches, UV light irradiation and thermal treatment; and air surface barrier discharge (SBD) plasma operated with three different discharge powers (P_d ; low P_d , 10 W; med P_d , 15 W; and high P_d , 20 W). Ambient gas was used as a feed gas.

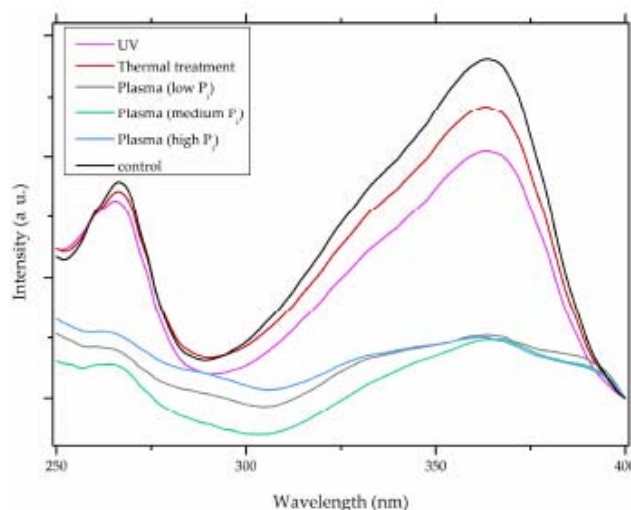


Figure 6. The comparison of aflatoxin B₁ (AFB₁) UV-Vis spectra after 8 min of exposure to heat treatment, UV irradiation and air surface barrier discharge (SBD) plasma operated with three different discharge powers (P_d ; low P_d , 10 W; med P_d , 15 W; and high P_d , 20 W). Ambient gas was used as a feed gas.

4. Conclusions

Mycotoxin contaminated food represents a significant and increasing threat to human health and an enormous burden for the global economy. Decontamination methods to tackle this problem are based on physical, chemical and biological principles. In spite of constant improvements, these methods can still suffer from a lack of mycotoxin removal efficiency, they can be environmentally harmful and economically unfavourable. With no doubt, the food industry continuously strives for more effective mycotoxin decontamination approaches.

One of the most promising new procedures to deactivate mycotoxins on food is CAP technology. On the laboratory level, it has been convincingly demonstrated that CAP efficiently kills fungi on the surface of food and destroys the mycotoxins that these organisms secrete. In favour over many of the traditional food decontamination methods, plasma-based decontamination methods are generally lower-cost and ecologically benign. Most importantly, plasma-based mycotoxin decontamination of food has been demonstrated significantly more efficient in both the mycotoxin degradation level and speed of decontamination in comparison to conventional decontamination methods, as presented for the case of one of the most toxic mycotoxins, AFB₁.

Before industrialization of CAP technology can be realised, the molecular mechanisms and kinetics of plasma-based mycotoxin decontamination should be better characterized in order to become standardized. For this reason, additional experimental work is needed to:

- Draw firm correlations between different plasma operating parameters and the specific reactive chemical species formed.
- Draw correlations between the composition of the plasma and the structure of the mycotoxin degradation products. As toxicities of the mycotoxin degradation products can be experimentally determined, in this way, the mycotoxin decontamination efficiency would be defined as well.
- Examine the effects of different plasma treatments on the quality of food products, for example on their nutritional value and organoleptic qualities.
- Design plasma-forming systems for efficient mycotoxin decontamination of various types and sizes of food products.
- Test if hybrid plasma-conventional systems for mycotoxin decontamination of food products can be even more effective.

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Chapter 3

Effective Fungal Spore Inactivation with an Environmentally Friendly Approach Based on Atmospheric Pressure Air Plasma

In Chapter 3, the challenges posed by dangerous mycotoxigenic mould contamination are addressed. Given that half of all fruit and vegetables harvested in tropical regions are lost due to fungal spoilage, it is not surprising that the environmental impact of fungal spoilage is catastrophic, resulting in serious health problems.

Current approaches to address this problem involve the use of different antifungal chemical agents, which themselves represent a burden for human health and the environment and should be urgently replaced with more effective environmentally friendly approaches. Thus, we reported the use of CAP as an alternative to the use of conventional disinfectants with fungicidal activity. To demonstrate the potential of the approach our method was analysed for its efficiency in comparison to the liquid chemical disinfection agent Virkon® for the case of *A. flavus* spores.

We demonstrated that the developed plasma decontamination method achieved fungal decontamination rates that were an order of magnitude faster compared to those achieved by the popular Virkon®. This result is explained through a comprehensive analysis of the reaction pathways initiated by CAP-generated RONS, which are ultimately responsible for the observed anti-fungal effect. However, the same efficiency was not observed with the fungal spores, which were indirectly exposed to the plasma-activated broth solution (PAB). This may be mostly attributed to the highly hydrophobic properties of the fungal spore surface, which prevent RONS to penetrate into the spore cell.

This chapter addresses thesis Objectives 1 and 2.

Regarding my contribution: I performed the CAP treatments of the fungal microbiological tests and the analysis of the liquid chemistry. I jointly wrote the paper with co-authors.

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Article

Effective Fungal Spore Inactivation with an Environmentally Friendly Approach Based on Atmospheric Pressure Air Plasma

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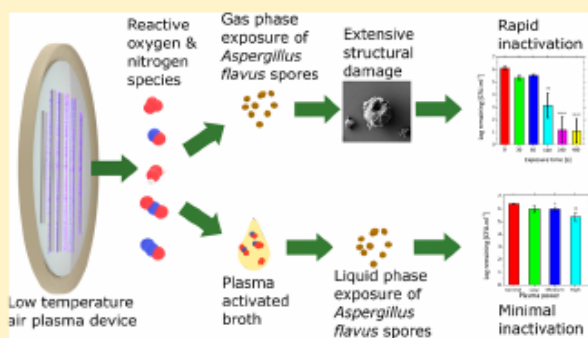
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Supporting Information

ABSTRACT: Fungal contamination of surfaces is a global burden, posing a major environmental and public health challenge. A wide variety of antifungal chemical agents are available; however, the side effects of the use of these disinfectants often result in the generation of toxic residues raising major environmental concerns. Herein, atmospheric pressure air plasma generated by a surface barrier discharge (SBD) is presented as an innovative green chemical method for fungal inactivation, with the potential to become an effective replacement for conventional chemical disinfection agents, such as Virkon. Using *Aspergillus flavus* spores as a target organism, a comparison of plasma based decontamination techniques is reported, highlighting their respective efficiencies and uncovering their underpinning inactivation pathways. Tests were performed using both direct gaseous plasma treatment and an indirect treatment using a plasma activated aqueous broth solution (PAB). Concentrations of gaseous ozone and nitrogen oxides were determined with Fourier-transform infrared spectroscopy (FTIR) and Optical emission spectroscopy (OES), whereas hydrogen peroxides, nitrites, nitrates, and pH were measured in PAB. It is demonstrated that direct exposure to the gaseous plasma effluent exhibited superior decontamination efficiency and eliminated spores more effectively than Virkon, a finding attributed to the production of a wide variety of reactive oxygen and nitrogen species within the plasma.



INTRODUCTION

Molds play an important role in various ecosystems and are used for the production of different types of foods and food supplements, beverages, enzymes, antibiotics, and other pharmaceuticals.^{1–3} However, molds are also responsible for a vast amount of global food spoilage and cause damage to a wide range of building materials and household surfaces. Additionally, they are also capable of producing a plethora of harmful organic metabolites. Many of the produced metabolites are proteins which can cause allergic reactions and respiratory diseases.⁴ Especially dangerous are food related fungal species, which are capable of producing toxic metabolites known as mycotoxins, many of which are known human carcinogens, neurotoxins, immunosuppressants, and endocrine disruptors.⁵

The occurrence of unwanted mold causes economic loss on a global scale; a challenge made even more pressing by the lack of a truly effective response measure to prevent it. Recently, several studies have indicated that the problems caused by fungal contamination are rising at an alarming rate due to improper agricultural practices and global climate change.^{6–8} Moreover, globalized economies demand the long distance transport of food from producer to consumer, meaning frequent changes in local climate and long storage times,

further exacerbating the issue.⁹ Current approaches to avoid mold contamination usually involve the use of groups of chemicals such as fungicides and disinfectants, where disinfectants are typically alcohol-, peracetic acid-, iodophors-, aldehydes-, chlorine-, peroxygen-based, or a mixture of these chemicals.¹⁰ For instance, a recently introduced and widely used liquid disinfectant known as Virkon is composed of a combination of oxone, sodium dodecylbenzenesulfonate and sulfamic acid plus various inorganic buffers. As a classical antimicrobial agent, it has a wide spectrum of disinfection activity, including effectiveness against fungal species.¹¹ As with other disinfectants, complete fungal inactivation using Virkon requires a significant exposure, dictating lengthy and complex decontamination procedures which increase the risk of inadvertent exposure and associated potential health risks. Following the inactivation procedure, the disinfectant must be discarded placing a burden on the environment and giving rise to the potential for toxic residues to remain.¹² Given the challenges posed by conventional liquid disinfection agents,

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new antifungal methods are urgently sought that offer an environmentally friendly “soft” approach, while ensuring sufficient fungicidal rates and minimal impact on the treated subject.¹³ Such approaches include nonthermal fungal inactivation methods including UV and pulsed light irradiation, gamma irradiation, and ozonation.^{14–18} The fungicidal effect of irradiation techniques is based on indirect action of electrons (e^-), hydroxyl radicals (OH), hydroxylperoxyl radicals (HO_2), hydrogen peroxide (H_2O_2) and other reactive species formed by radiolysis of cellular water, which leads to lethal structural damages in fungal cells, including DNA strand breakage, cell membrane rupture, or mechanical impairments of cell walls.¹⁴ Ozone (O_3) is highly potent oxidizing agent. With its capability to diffuse through membranes, it is able to attack various constituents in cell membranes, cell walls, and cytoplasm.¹⁹ Recently, a novel nonthermal method known as atmospheric pressure plasma treatment was introduced.²⁰

Cold atmospheric pressure plasma (CAP) technology represents a promising new and green approach to fungal inactivation.^{21,22} It has already been proven to be an efficient tool for the inactivation of microbial pathogens and other toxic agents from surfaces and food products, the degradation of pollutants in potable water, treatment of cancer cells and tumors, as well as a host of other applications spanning the domains of environment science, biology, and nanotechnology.^{23–28} Yet, to date, few studies have explored fungal inactivation with air based CAP.

In principle, the plasma state of matter refers to ionized gas with unique physical and chemical properties. CAP is generated by adding energy (e.g., through an applied electric field) to a gaseous state, resulting in the creation of a mixture of ground and excited state atoms and molecules, electrons, ions, free radicals, and UV photons.²⁹ The generation of nonequilibrium atmospheric pressure plasma results in the production of a wide spectrum of reactive oxygen and nitrogen species (RONS) at low temperatures. These species play an important role in the biological activity of CAP due to their high oxidative potential. The most critical among them are atomic oxygen (O) and nitrogen (N), OH, superoxide (O_2^-), HO_2 , O_3 , H_2O_2 , nitrogen oxides (NO_x), and peroxyne ($ONOO^-$).³⁰

A promising alternative approach compared to directly applying the plasma to a target surface for the purpose of disinfection is to use a “plasma activated liquid”. In this modality, CAP is applied to a liquid which initiates a wide variety of interactions between the gaseous CAP species and liquid media, resulting in the diffusion of gaseous RONS into liquid and the creation of unique liquid chemistries that have shown a broad spectrum of biological effects.³¹ The proven antimicrobial activity of both gaseous and liquid RONS originates from the damage they cause to the microbial cell wall and/or membrane, subsequently enabling further reactive species to enter the cell and damage the intercellular components such as organelles and important biomolecules (DNA, RNA, and proteins).^{26,32,33}

Some studies have indicated that CAP treatments can be effective for the inactivation of various fungal species from the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Cladosporium*,^{34–38} compared to other nonthermal decontamination approaches such as UV irradiation and ozonation. For instance, a 5.48 log reduction for *Aspergillus flavus* and a 5.2 log reduction for *Aspergillus parasiticus* were achieved after a 5 min exposure to an atmospheric pressure fluidized bed air plasma.³⁴

Furthermore, greater than 4 log of *Penicillium digitatum* spores were inactivated after a 7 min exposure to an atmospheric pressure oxygen CAP system.³⁹ Despite encouraging results from these reported studies, the reported CAP systems cannot be easily applied for real-world large-scale processing or implementation in an industrial environment. Moreover, the detailed physical-chemical pathways at play in the plasma and its biological outcomes in terms of fungal inactivation remain elusive, requiring significantly more investigation in the technology before it could hope to see widespread industrial adoption. In order to overcome these knowledge gaps, this study focuses on the inactivation of fungal spores using a surface barrier discharge (SBD) CAP system employing only ambient air. One of the main advantages of SBD is its scalability. As previously demonstrated, uniform plasma can be generated over electrode area of 100×100 mm and can be scaled up or down to required size.⁴⁰ In this study, *Aspergillus flavus* was chosen as it poses a particular hazard to humans due to its ability to produce highly carcinogenic mycotoxins, known as aflatoxins.⁴¹ The SBD source was used to directly treat fungal spores and to generate plasma activated aqueous broth solution (PAB). The fungicidal efficiency of both direct CAP treatment and application of PAB were compared with a standard 1% Virkon solution. A host of plasma and liquid diagnostic techniques were employed in order to link the physical and chemical properties of both the gaseous plasma effluent and the composition of the PAB to the biological effects observed. Ultimately, the results of this study indicate that direct air CAP treatment is an extremely effective antifungal agent, providing key advantages over competing approaches by offering an ability to generate high densities of RONS directly at the point of need, using only ambient air and very little electrical power. It is these key attributes that will greatly facilitate the widespread implementation of CAP technology for fungal decontamination, ultimately serving to reduce the need for potentially toxic liquid disinfectants and thus minimizing the environmental burden associated with fungal inactivation.

MATERIALS AND METHODS

CAP System. The highly scalable CAP system used in this investigation was similar to that reported by Ni et al.,⁴² comprising of a plasma generating electrode unit fabricated from a 100 mm diameter, 1 mm thick quartz disc with aluminum electrodes adhered either side. On one face of the quartz disc, the electrodes were connected to a high voltage power source and coated in epoxy resin to prevent plasma formation. On the opposing side of the quartz disc, a counter electrode was created by connecting the strips to the power source ground. On application of a high voltage AC signal, plasma formed around the edges of the grounded electrode. Figure 1(a) shows a schematic representation of the plasma source and 1(b) shows a photograph of the system operating at an applied voltage of 10 kV. To provide the high-voltage, a homemade power source capable of generating voltages ranging from 5–15 kV peak to peak at 40 kHz was employed. It is well-known that air plasma produced using a SBD under ambient conditions generates an abundance of biologically active RONS. The particular composition of the discharge effluent is strongly influenced by the discharge properties, including the applied voltage and dissipated power.

In this investigation, the CAP system was operated under three different dissipated power regimes obtained by varying

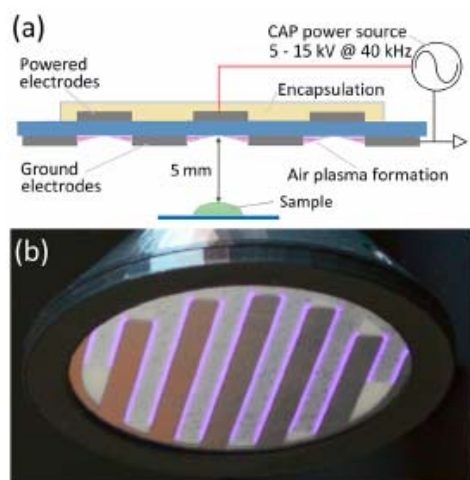


Figure 1. (a) Schematic of air SBD plasma system used for fungal spore inactivation; (b) photograph of CAP system operating under high-power conditions.

the applied voltage. Under low power conditions, with a power of 0.79 W/cm^2 the gas phase chemistry in the headspace between the active discharge layer and the sample was found to be dominated by reactive oxygen species (ROS). Under high power conditions, with the power set at 1.62 W/cm^2 , the gas composition was dominated by reactive nitrogen species (RNS). A medium power condition was identified, with power set to 1.24 W/cm^2 , simultaneously generating both ROS and RNS chemistries.

Fungal Spore Preparation. *A. flavus* spores (strain EXF-532) were provided by the Mycosmo microbiological bank (Ljubljana, Slovenia) and used in all investigations reported here. To produce spores, molds were first grown on fresh potato dextrose agar (PDA; VWR Chemicals) plates for approximately 7 days at 25°C . Following successful growth, a stock suspension of spores was prepared by flooding the PDA plates with an aqueous solution of tryptic soy broth (TSB; Biolife) including the addition of 0.05% Tween 80 (Acros Organics). Following addition of the broth solution, spores were gently scraped from the surface of the newly formed mycelia using a sterile loop. To achieve the highest possible homogeneity, the stock spore suspension was mixed thoroughly using a sterile injection needle. To determine the concentration of spores in solution, an optical microscope was used with the help of an improved Neubauer Brightline hemocytometer (BRAND).

CAP Treatments. In this study, the plasma source was used to generate a broad spectrum of long-lived RONS, as shown in Figure 2(a), these species were applied to the sample using two alternate approaches; one involving the direct exposure of spores to the gas phase effluent of the plasma, shown in Figure 2(b) and the other involving the plasma treatment of aqueous broth solution and subsequent application of the PAB to the spores, shown in Figure 2(c). To accommodate these different tests, one-third of the prepared spore solution was deposited on to glass coverslips to give a surface concentration of 10^6 spores and allowed to dry for 5 min. These samples were used to assess the effectiveness of a gas phase CAP treatment. The experimental approach used to directly expose fungal spores is presented in Figure 2(b). To assess the potential of PAB for

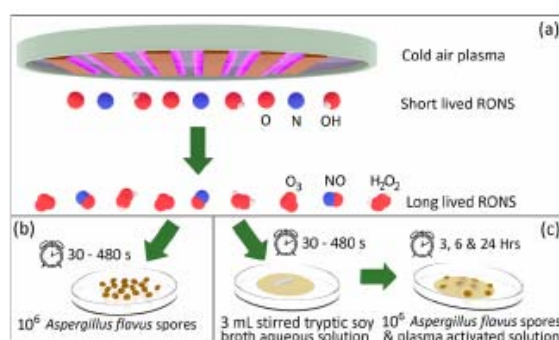


Figure 2. Diagrammatic representation of CAP treatment methodologies: (a) CAP system and the key short- and long-lived plasma generated RONS; (b) direct CAP treatment of *A. flavus* spores; (c) CAP treatment of aqueous tryptic soy broth solution and subsequent exposure of *A. flavus* spores to plasma activated broth solution (PAB).

inactivation of fungal spores, 3 mL of sterile aqueous broth solution with 0.05% Tween 80 was exposed to plasma under the same conditions used in the gas phase treatment scenario. The resulting PAB was subsequently added to the spore solution for the purpose of decontamination, as presented in Figure 2(c). Finally, the remaining third of the prepared spore solution was reserved to assess the decontamination efficacy of Virkon, a commonly used disinfection agent with strong fungicidal action providing a valuable benchmark from which to compare respective plasma treatments.

During CAP treatments, the SBD electrode was positioned on top of an open Petri dish lid containing either a glass coverslip hosting fungal spores or the aqueous TSB broth solution. In all cases, the distance between the active discharge and the sample was fixed at 5 mm. For all tests involving liquid treatment, the CAP system was placed on a magnetic stir plate, and the sample continually stirred at 225 rotations per minute.

To treat the spores deposited directly on to a glass coverslip, CAP was applied at low, medium and high power for time durations of 30, 60, 120, 240, and 480 s. Following CAP exposure, the coverslips were washed with 1 mL of sterile broth solution. Serial decimal dilutions of this solution were prepared and plated on to fresh PDA plates followed by incubation for 2 days at 25°C . Following incubation, colonies were counted and converted to logarithmic values. For experiments involving PAB, the solutions including both spores and PAB were transferred to microcentrifuge tubes and incubated for 3, 6, and 24 h at 37°C in shaking incubator at 150 rpm. Following incubation, serial decimal dilutions of the spore samples were plated on to fresh PDA plates and colonies forming were counted. To provide a comparison with conventional methods, the spore solution was also exposed to a 1% aqueous solution of Virkon (Du Pont). This was achieved by spraying the Virkon on to spores deposited on a glass coverslip for 480 s, which is equivalent to the longest plasma exposure time. In the case of spore suspension treatment, spores were exposed to Virkon for 3, 6, and 24 h. In all cases, tests were performed in triplicate to provide a statistically robust comparison.

Metabolic Activity (XTT Test) Of Spores. After treatment, the metabolic activity of the spores was assessed using a Mitochondrial activity test (XTT test). This was achieved by adding a 10 mL solution of PBS (Sigma-Aldrich) containing a

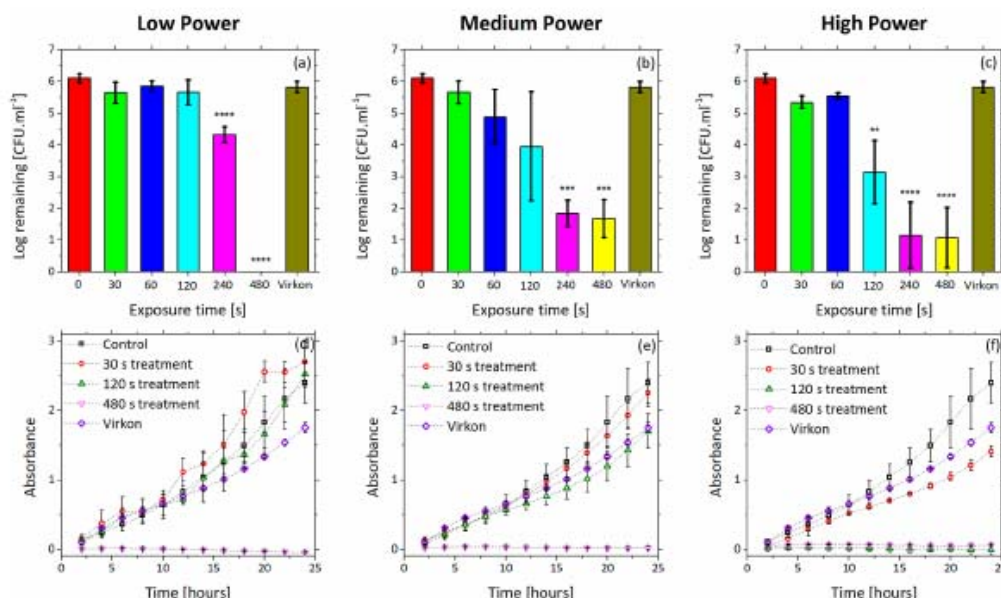


Figure 3. CAP effluent treatment of *A. flavus* spores: (a), (b), and (c) log numbers of new grown spore units after low, medium, and high power plasma treatment with a comparison provided against a 1% Virkon solution. The initial concentration of spores for (a), (b), and (c) was 1.3×10^6 CFU/mL. An one way ANOVA was performed between treated samples and control samples (0 s). *P*-values = 0.0332 (*); 0.0021 (**); 0.0002 (***) < 0.0001 (****); (d), (e), (f) metabolic activity of spores exposed to low, medium and high power plasma and a 1% Virkon solution comparison.

10 mM concentration of XTT (2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; Alfa Aesar). Prior to the test, a 1 μ L of menadione (2-methylnaphthalene-1,4-dione; Acros Organics) prepared in 100% acetone (VWR Chemicals) was added to the solution. For the test, 50 μ L of the sample, containing a concentration of 10^6 spores/mL, was placed in a 96-well plate and mixed with 50 μ L of saline solution and 50 μ L XTT reagent. The well plates were then incubated at 37 $^{\circ}$ C for 2 h in the dark to form the colored XTT formazan product. The absorbance at 450 nm wavelength was measured after 4, 8, 12, 16, 20, and 24 h.

Scanning Electron Microscopy. Mold spores exposed to both the CAP gas phase effluent and PAB were first submerged in a 2.5% glutaraldehyde fixative solution and incubated overnight. The samples were then dehydrated in a series of increasing ethanol concentrations, finally being placed in 100% ethanol. After that, samples were dried in hexamethyldisilazane (HMDS). Once in 100% HMDS, spores were placed on aluminum SEM holders and coated with a 6 nm platinum layer to prevent charging effects during analysis. SEM (Jeol JSM-7600F) was performed at 2 kV.

Plasma Activated Liquid Analysis. Immediately following the generation of PAB, the pH level was measured using a Hanna instruments HI 98129 combined pH and conductivity probe. To further understand the chemical composition of the activated liquid, colorimetric tests were performed to measure hydrogen peroxide (H_2O_2), nitrite (NO_2^-), and nitrate (NO_3^-) concentrations. To determine the presence of H_2O_2 , a ferric-xylenol orange complex (xylenol orange, sorbitol and ammonium iron sulfate; Sigma-Aldrich) was added to the treated solution and the absorbance measured at 560 nm. NO_2^- concentrations were evaluated using a standard Griess reagent assay (Griess reagent system; Promega) examining

absorbance values at 540 nm. The NO_3^- concentration was determined in plasma treated deionized water by measuring the absorbance at 200 nm followed by comparison with the standard calibration curve. The measurements of NO_3^- in plasma treated deionized water were compared against the values determined in PAB using test stripes (Quantofix Nitrate/Nitrite test strips).

Fourier-Transform Infrared Spectroscopy Analysis.

Generation of CAP using an SBD produces a wealth of long-lived chemical species that are able to travel well beyond the confines of the visible discharge. To analyze the long-lived gas phase species under low, medium and high power operating conditions the SBD electrode unit was placed within a sealed enclosure, and the effluent from the system was drawn through a Fourier-transform infrared (FTIR) gas cell (multipass design with 16 m path length) using a small vacuum pump (<1 l/min). A Jasco FT/IR-4000 spectrometer with a mid-IR optical bench from 7800 to 500 cm^{-1} was used to record the resulting IR absorption spectrum. Measurements were taken with a resolution of 4 cm^{-1} and each spectrum comprised of 25 individual acquisitions averaged. To obtain the concentration of relevant species from the absorption spectra, a fitting procedure based on a comparison of peak area against standard reference profiles obtained from the Pacific Northwest National Laboratory (PNNL) library was implemented.⁴³ The standard reference data was collected at 1 part-per-million-meter (ppm-meter) resolution at 296 K with a path length of 1 m. To obtain the concentration, *C*, of a particular molecule from the absorption spectra, the equation $C = A / (B \cdot l)$ was used, where *A* is the area of the FTIR peak of a particular species, *B* is the standard peak area, and *l* is the difference between the experimental gas cell path length and the path length used to obtain the reference data.

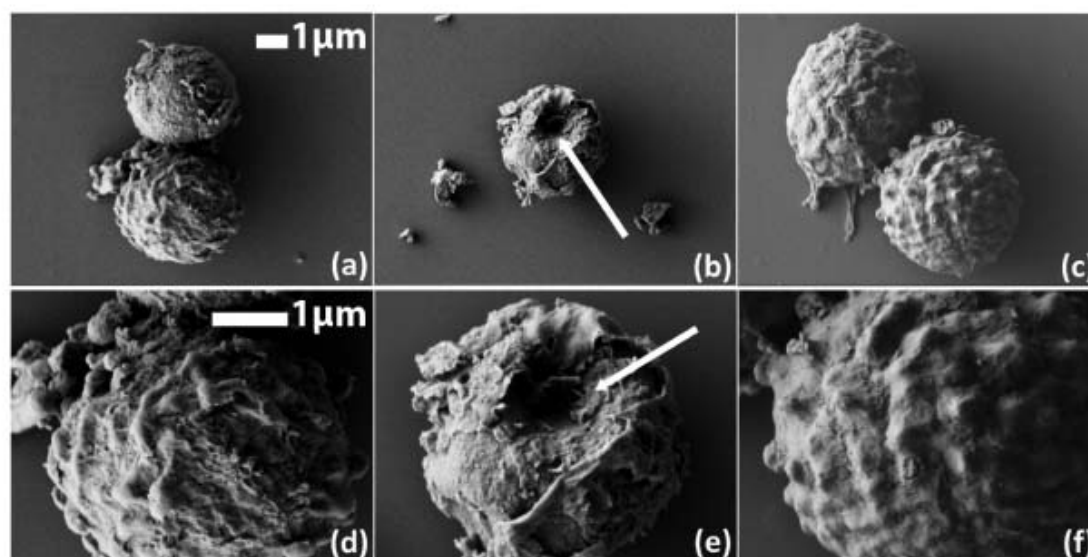


Figure 4. Characteristic scanning electron microscopy images of *A. flavus* spores after exposures to the direct CAP treatment and a 1% Virkon solution under 10 000 $\times$ magnification ((a), (b), (c)) and 25 000 $\times$ magnification ((d), (e), (f)): (a) and (b) untreated sample (control); (b) and (e) sample treated with CAP at high power for 120 s with marked plasma produced spore morphology damage; (c) and (f) sample treated with 1% Virkon for 480 s.

Optical Emission Spectroscopy. Optical emission spectroscopy (OES) was used to obtain an indication of the nature of excited species produced in the discharge layer and to assess the rotational and vibrational temperature of the plasma. A fiber optic coupled spectrometer (Ocean Optics HR2000+) with a spectral resolution of ~ 0.5 nm was employed to record the emission spectra from the discharge under low, medium, and high power operating conditions. To obtain the rotational and vibrational temperature the recorded emission spectra was compared against synthetic spectra of the Nitrogen Second Positive series, generated using the commercial software Specair, and the best fit temperature obtained.

Sample Temperature Measurements. A thermocouple probe was used to measure the sample temperature as a function of treatment time. The probe was positioned on the glass coverslip or within the liquid sample and the temperature recorded at 30 s intervals throughout plasma exposure.

Statistical Analysis. Three replicate samples were conducted for each time point in the experiment. One way and two way ANOVA was used to evaluate the significant difference between the experiment groups (GraphPad Prism 7).

RESULTS AND DISCUSSION

Antifungal Effect of Gaseous Plasma Treatment. *A. flavus* spores deposited on glass coverslips were directly exposed to the CAP effluent under the plasma generation conditions outlined in the experimental methods section. The antifungal effect of the treatment is highlighted in Figure 3. From the figure it is clear that a 480 s CAP treatment resulted in considerable spore inactivation, exceeding a 5 log reduction under both the low and high power conditions (Figure 3(a) and (c)). In general, the log reduction increased with exposure time and applied power. The highest power condition gave the most rapid level of inactivation, reaching a 2.19 log reduction after just 120 s of exposure (Figure 3(c)). Conversely, the low

power condition showed a very little effect until 240 s, at which point a rapid increase in the level of inactivation occurred, ultimately achieving complete inactivation (defined as <1 log surviving CFU) within 480 s (Figure 3(a)). To provide a comparison of CAP inactivation efficacy against a widely used liquid decontamination agent, spores were spread on a glass coverslip and exposed to a 1% Virkon solution for a total of 480 s. Under such conditions, no reduction in viable spores was observed (Figure 3(a)–(c)). Such results indicate that gas phase plasma treatment is considerably more effective than a standard Virkon solution. These findings were supported by the XTT analysis, which highlights a minor decrease in the metabolic activity of Virkon treated spores; in contrast, plasma treated spores exhibited significantly reduced activity at considerably shorter exposure times (Figure 3(d)–(f)).

Insight into the mode of inactivation during CAP exposure can be obtained from SEM images of the treated spores. The typical cases of untreated, CAP treated, and Virkon treated spores under two different levels of magnification are presented in Figure 4. As seen in Figure 4(b) and 4(e), a high power plasma treatment resulted in considerable structural damage to the *A. flavus* spores. It was observed that a large percentage of the treated spores exhibited similar levels of structural damage and were surrounded by considerable cell debris. In contrast, Virkon treated spores, presented in Figure 4(c) and (e), showed little difference from the untreated spores.

To explain the observed decontamination results and aid in identification of the most likely underpinning inactivation pathways involved in gas phase CAP treatment FTIR, OES and temperature measurements were made. Initially, to rule out thermal effects, a thermocouple was placed at a position 5 mm beneath the SBD electrode. In all cases thermal equilibrium was reached within 120 s of CAP exposure and temperatures of 35 $^{\circ}\text{C}$, 48 $^{\circ}\text{C}$, and to 52 $^{\circ}\text{C}$ were measured for the low, medium, and high power conditions, respectively. While these temperatures are not insignificant, studies have demonstrated that such temperatures are incapable of achieving a significant

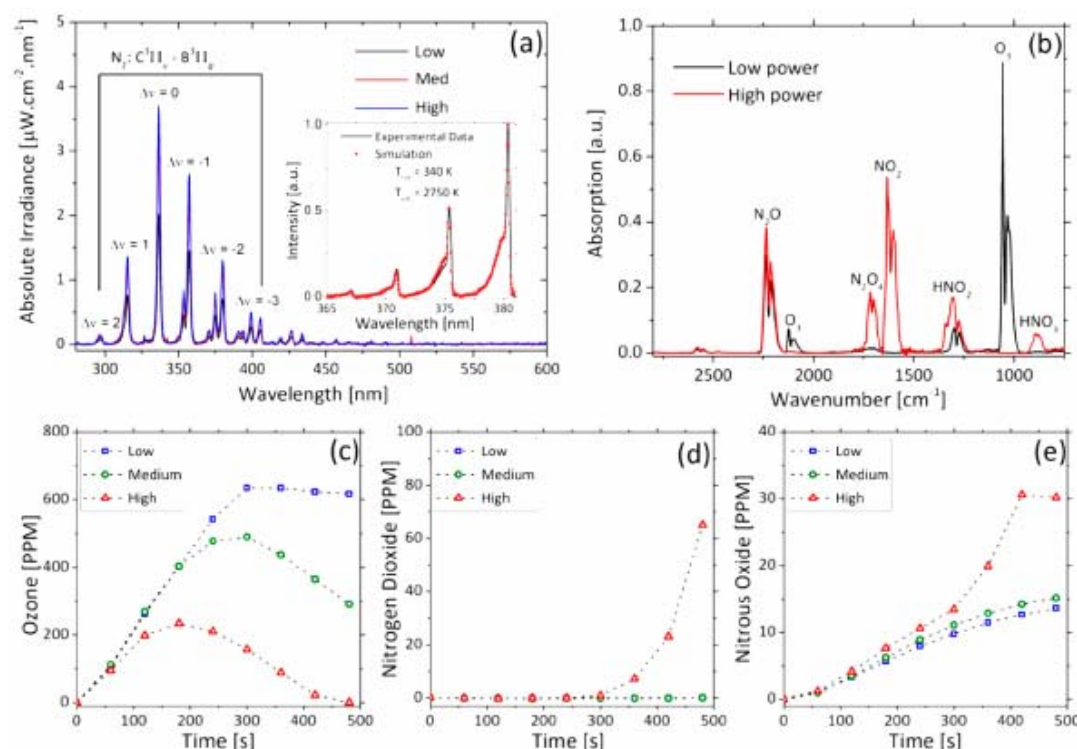


Figure 5. Gas chemical composition of plasma gas phase produced by air SBD operated at low, medium and high dissipated power; (a) OES and vibrational and rotational temperature of electrons; (b) FTIR spectra of long-lived species; (c) ozone content; (d) nitrogen dioxide content; (e) nitrous oxide content.

level of mold spore inactivation on the time scales considered in this study.⁴⁴ Such results provide a strong indication that thermal effects are not the primary inactivation mechanism. To gain an insight into the composition of the plasma, OES was employed to capture the optical signature of the excited species within the discharge layer. The overlaid emission spectra of the discharge operating under low, medium and high power conditions is displayed in Figure 5(a). The spectra reveal that in all cases the excited nitrogen second positive series was the dominant emission, appearing between 290 and 410 nm. No other significant emission lines were observed within the 280–600 nm wavelength range regardless of operating power. Given the emission data was calibrated for intensity it is possible to conclude that the light intensity produced by the CAP over the UVC, UVB, and UVA ranges is well below that known to have an impact on the growth of *A. flavus* spores, with levels around $\mu\text{W}\cdot\text{cm}^{-2}\cdot\text{nm}^{-1}$. Therefore, UV effects from the CAP device can also be neglected, a finding consistent with previous studies.⁴⁵

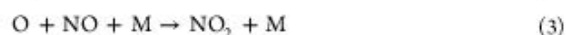
Further analysis of the second positive system makes it possible to obtain an estimate of the gas temperature within the discharge layer. It is well-known that under the atmospheric pressure conditions the rotational temperature of excited nitrogen is in thermodynamic equilibrium with the background gas. By comparing the recorded emission spectra against a synthetic spectra calculated at a known temperature an approximation of the gas temperature was obtained. As shown in the inset of Figure 5(a), the best-fit temperature at the highest plasma power was found to be 340 K with an error

of ± 25 K. This value corresponds well with the thermocouple measurements made at the sample location.

Beyond UV and thermal effects, it is well-known that air plasma generated at atmospheric pressure creates highly reactive short-lived species, for example, O, OH, N, and O_2^- , which diffuse away from the plasma region, rapidly reacting to form a variety of longer-lived RONS, including O_3 , H_2O_2 , nitric oxide (NO), and other NO_x .⁴⁶ The density of some of these species can be quantified from FTIR absorption measurements obtained during plasma operation, as shown in Figure 5(b). Under low power conditions, it can be seen that O_3 is the primary long-lived product of the discharge. O_3 is primarily produced in the discharge layer through a three-body reaction involving atomic oxygen (1).



Conversely, under high power conditions, the composition of the discharge effluent is dominated by NO_x including nitrous oxide (N_2O) and nitrogen dioxide (NO_2) (Figure 5(b)). The origins of the transition between O_3 dominated to NO_x dominated discharge chemistries is 2-fold: first, as discharge power increases, the rate of thermal decomposition of O_3 rapidly increases.⁴² Second, an increased power yields more NO which acts to poison O_3 production by simultaneously reducing O_3 through direct decomposition to NO_2 and through the consumption of O, a key precursor for O_3 formation (Reactions 2 and 3).⁴⁷



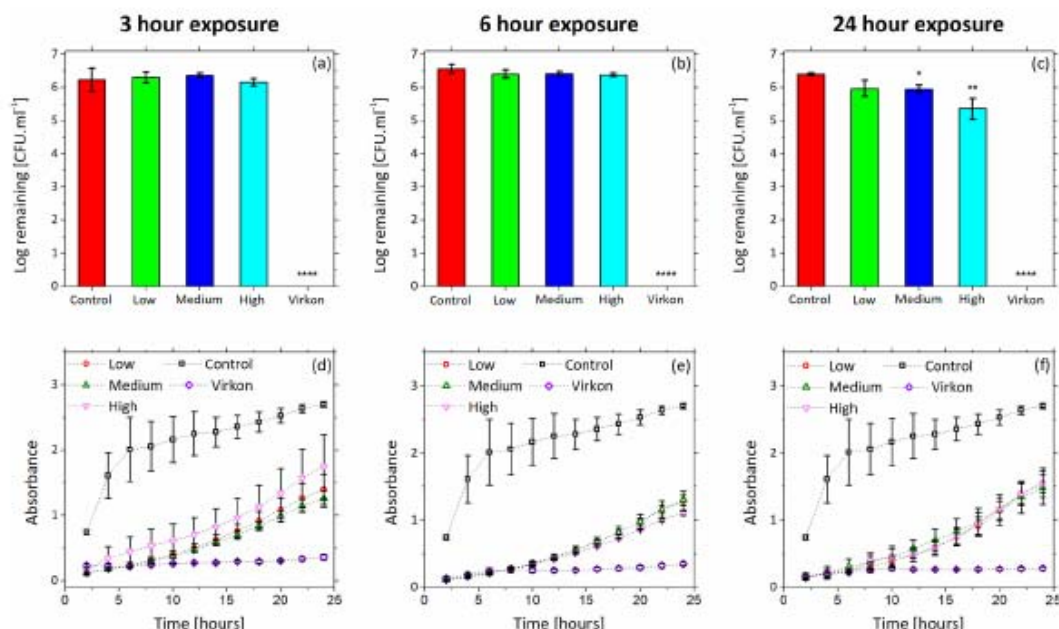


Figure 6. Exposure of *A. flavus* spores to plasma activated broth (PAB) generated under low, medium and high power conditions for 480 s with a comparison provided against a 1% Virkon solution: (a), (b), (c) log numbers of new grown spore units after exposure to PAB and Virkon for 3, 6, and 24 h. The initial concentration of spores for (a), (b), and (c) was 2.1×10^6 , 3.8×10^6 and 2.5×10^6 CFU/mL, respectively. An one way ANOVA was performed comparing results of treated samples to control (0 s). *P*-values -0.0332 (*); 0.0021 (**); 0.0002 (***); < 0.0001 (****); (d), (e), (f) metabolic activity of spores exposed to PAB and Virkon for 3, 6, and 24 h.

Time-resolved FTIR measurements reveal the evolution of O_3 , NO_2 , and N_2O in the discharge effluent and clearly demonstrate the poisoning effect (Figures 5(c–e)). Under high power operation, O_3 formation is rapidly inhibited as the discharge reactor reaches thermal equilibrium and the nitric oxide concentration increases. As O_3 poisoning occurs, a significant increase in NO_2 is observed. Finally, it is worth pointing out that several key RONS present in the plasma effluent may not be measured using FTIR, either because they do not actively absorb infrared light, or because they react to form alternative species between the discharge chamber and the FTIR gas cell, nitric oxide being a prime exemplar.

The biological effects of RONS are well-known. O_3 is a powerful oxidant; it can oxidize organic matter either directly or through OH produced during the decomposition of O_3 . First, it interacts with double bonds of unsaturated lipids and the sulphhydryl groups of membrane enzymes. This damage causes disruption of cell permeability, leading to leakage and oxidation of intercellular contents and biomolecules such as nucleic acids and eventually the lysis of the cell.⁴⁸ Another potent oxidant produced by CAP is H_2O_2 , which has inactivation mechanisms similar to those of O_3 . Generally, cytotoxicity caused by H_2O_2 begins with its penetration into the cell and then the production of OH. OH is an extremely potent oxidizing agent which can be created from the decomposition of both O_3 and H_2O_2 . Its primary targets are cell walls and membranes, therefore all the compounds composing of these two structures. The mechanism of OH reaction with lipids refers to its H-abstraction from the unsaturated carbon bonds of the fatty acids, ending in lipid peroxidation.^{49–53} Simultaneously, OH also reacts with membrane proteins by H-abstraction from the α -carbon of the peptide bonds between chain peptide-linked amino acids,

resulting in peroxidation of proteins. In comparison with the direct activity of O_3 , the mechanism of OH inactivation is less selective.⁵⁴ Given that the SEM images in Figures 4(b and e) demonstrate that spores subjected to CAP treatment exhibit significant structural changes, it is highly probably that ROS species have an etching effect on the spore wall, creating an entry point for RONS to react further with the intracellular contents.

Some previous studies have hypothesized that the interaction between plasma generated RONS and fungal spores is one of the most likely chemical pathways for inactivation.^{37,55} When CAP decontamination efficacy is compared against the most frequently used methods for fungi decontamination, conventional UV irradiation or O_3 treatment, the CAP method is significantly more effective. Recent studies of conventional approaches demonstrated that UVC irradiation achieved a modest 2.8 log reduction of *Penicillium expansum* spores after a 6 min exposure at a light intensity of 3.3 kJ/m^2 .⁵⁶ Similarly, gaseous O_3 was found to be even less effective, with a 3 log reduction achieved for various spores types exposed to 35 ppm of O_3 for 20 min.¹⁷ Since the low power CAP treatment accomplished a high inactivation level within a short time frame, it is reasonable to conclude that O_3 alone is not solely responsible. Therefore, the simultaneous exposure to multiple RONS, UV and elevated gas temperatures are likely to result in synergistic effects that accelerate spore inactivation in the presence of plasma.

Antifungal Effect of Plasma Activated Broth. Recently, a considerable research effort has been focused on the use of plasma treated liquids for microbial inactivation applications. Examples include the inactivation of *Escherichia coli*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Candida albicans*, etc.^{42,57} Using a liquid as a carrier for reactive plasma

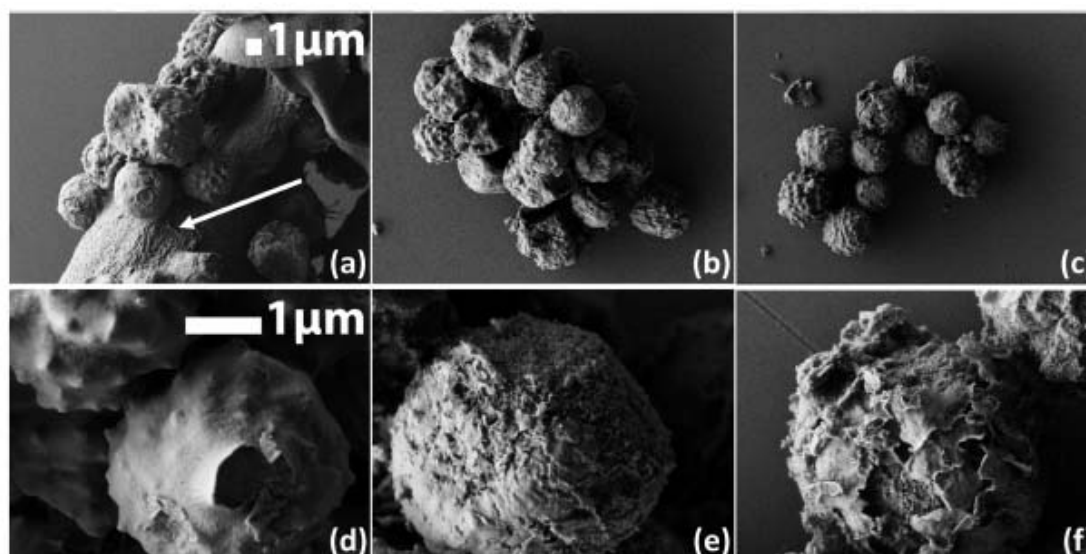


Figure 7. Characteristic scanning electron microscopy images of *A. flavus* spores after a 6 h exposure to PAB and a 1% Virkon solution under 5000 x magnification ((a)–(c)) and 25 000× magnification ((d)–(f)): (a) and (c) sample incubated in untreated aqueous TSB broth solution (control) with marked mycelia; (b) and (e) sample treated with PAB; (c) and (f) sample treated with 1% Virkon solution.

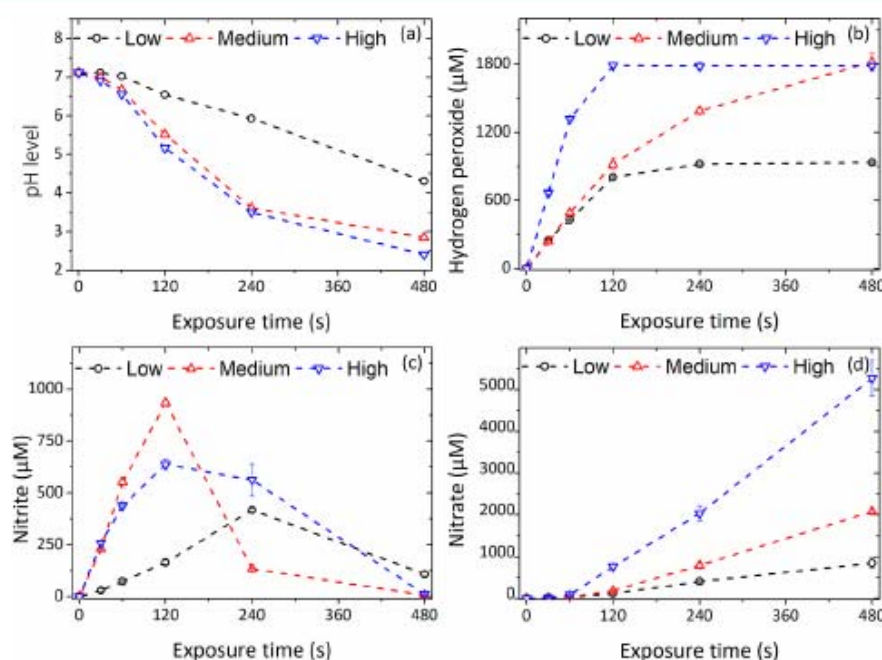


Figure 8. Liquid phase chemistry of PAB. Created using CAP under low, medium and high dissipated powers. A two way ANOVA was performed comparing results of treated samples to control (0 s); (a) pH values of PAB; (b) concentration of hydrogen peroxide. All treated samples were significantly different compared to control ($P \leq 0.001$); (c) concentration of nitrite. All treated samples were significantly different ($P \leq 0.001$) with exceptions of those treated for 30 s with low power plasma and 480 s with medium and high power plasma; (d) concentration of nitrate. Significant differences ($P \leq 0.001$) occurred after 240 s of exposure to low and medium power plasma, and after 120 s of exposure to high power plasma.

species overcomes some drawbacks associated with a gas plasma treatment including an ability to treat highly porous materials with complex morphologies and enabling the treatment of highly temperature sensitive systems or environments where gaseous plasma cannot be directly used. Another comparative advantage of this treatment modality is the long-

lived nature of the PAB (up to 1 week),⁵⁸ which can be used when needed, without the need to create a plasma.

To assess the potential of PAB for fungal inactivation, a PAB solution was prepared and subsequently used to expose *A. flavus* spores for varying lengths of time. The inactivation results are presented in Figure 6(a–c). Clearly, the inactivation

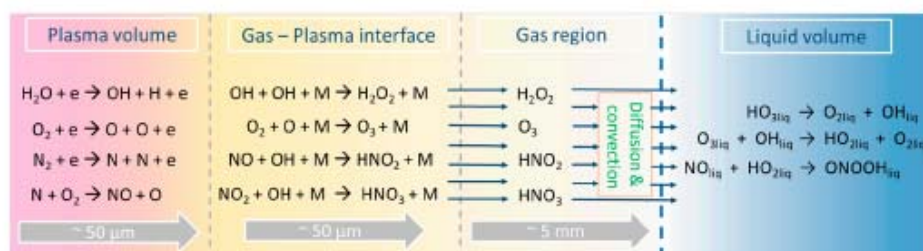


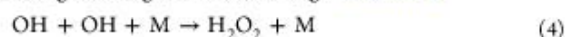
Figure 9. Schematic of main reactions during the CAP treatment of liquid.

efficacy of PAB is significantly lower than the corresponding gas phase treatment. No significant reduction in spore count was observed with the exception of 24 h exposure to PAB created using high power plasma conditions, yielding a 1.04 log reduction. In contrast, exposure to a 1% Virkon solution led to complete inactivation under all conditions investigated. Interestingly, the metabolic activity of PAB treated spores significantly decreased under all treatment conditions, reaching a value two times lower compared to the control (Figure 6(d–f)). Given that previous studies have demonstrated the high antimicrobial potential of PAB, the results from Figure 6 are unexpected since it would be reasonable to assume that similar inactivation pathways are at play. To unravel this, the untreated, PAB treated and Virkon treated *A. flavus* spores were analyzed by SEM after a 6 h incubation in fresh aqueous TSB solution (Figure 7). From the observed morphology, some minor differences were detected between the untreated (Figure 7(a) and (d)) and PAB treated spores (Figure 7(b) and (e)); however, spores exposed to Virkon exhibit significant structural damage (Figure 7(f)). Nevertheless, the growth and the development of hyphae and mycelia of *A. flavus* exposed to PAB treated with high power CAP was decreased or arrested (Figure 7(b) and (e)). In comparison, the formation of mycelia can be observed at untreated sample (Figure 7(a)).

In order to understand the results, the nature of PAB must be considered. CAP interaction with liquid induces a wide range of physicochemical changes. The creation of aqueous phase chemical species is strongly influenced by the type of discharge, its underpinning physical processes and the chemical composition of the surrounding environment, such as the type of gas and liquid used. Mass transport from the plasma phase, through the separating gas layer to the liquid phase, leads to the generation of a wide variety of reactive species in the bulk liquid. In this study, the liquid chemistry is mostly affected by long-lived species despite the relatively short distance between the plasma and the liquid surface, as the most reactive chemical species are confined to the visible discharge layer.⁴⁶ To follow the kinetics of the aqueous phase reactive species, the concentrations of H_2O_2 , NO_2^- , NO_3^- and pH were measured as a function of CAP treatment time (Figure 8).

In Figure 8(b) we suggest that CAP treatment induces the formation of H_2O_2 in the broth solution. The increase in H_2O_2 concentration is highly dependent on the discharge power employed. For low and high power treatment conditions, the H_2O_2 concentration is increased up to an equilibrium or saturation level. Conversely, PAB created under medium power operation CAP operation showed a different trend, with a continual increase. The highest concentration of H_2O_2 was measured after 480 s in the medium power case, at over 1800 μM . Based on recent plasma modeling studies,^{59,60} it is

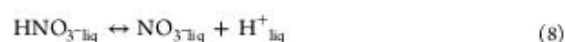
expected that H_2O_2 present in the liquid phase was either generated in situ or created in the humid air surrounding the plasma generating electrode, through Reaction 4.



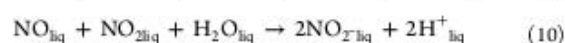
Considering the reasonably large air gap between the plasma layer and liquid surface, it is highly unlikely that OH produced in the discharge will reach the liquid interface in any significant concentration. Consequently, the direct formation of H_2O_2 at the gas–liquid interface will be minimal (Figure 9). Several studies have used electron spin resonance techniques to measure OH directly in the liquid phase,⁶⁰ this has been attributed to in situ generation pathways involving plasma generated RONS that are capable of transport across the dividing gas layer, pertinent reactions include Reactions 5 and 6:



The presence of NO_2^- was measured using the Griess reagent method. Figure 8(c) demonstrates that regardless of the plasma power the NO_2^- concentration initially increased with treatment time, reaching a peak, followed by a significant decrease with further treatment. Conversely, the NO_3^- displays an almost linear increase with treatment time under all power conditions (Figure 8(d)). Both NO_2^- and NO_3^- in the CAP treated broth solution are created through numerous reaction pathways. Mass transport of nitrous acid (HNO_2) and nitric acid (HNO_3) from the gas phase to the liquid provides the primary reactants to form NO_2^- and NO_3^- . Once in the liquid, they are hydrolyzed to form NO_2^- , NO_3^- , and H^+ (Reactions 7 and 8):⁶⁰

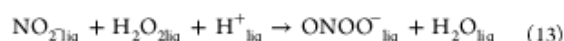
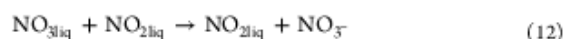
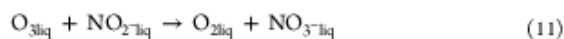


Another important pathway involved in the creation of these ionic species involves NO and NO_2 generated in the gas phase downstream of the discharge layer (Reactions 9 and 10):⁶⁰



Eventually, the concentration of H^+ rises to a level where hydrolysis reactions are inhibited, contributing to a reduction of pH,^{53,60} which can also be observed in the presented study. The pH level decreased over time under all examined power conditions, reaching a pH of 2.4 after 480 s of exposure (Figure 8(a)) in the highest power case. The results presented

in Figure 8 and the observed drop in NO_2^- concentration can be explained by considering its reaction with dissolved O_3 and NO_3^- to become NO_3^- , Reactions 11 and 12.⁶¹ Under the observed acidic conditions, NO_3^- is also generated through the reaction of NO_2^- with H_2O_2 , forming short-lived ONOO^- via Reaction 13.^{33,60}



Based on the liquid chemistry analysis detailed in Figure 8, the low rate of fungal spore inactivation observed in Figure 6 remains somewhat surprising. The PAB contained significant amounts of RONS such as H_2O_2 , NO_2^- and NO_3^- . Moreover, it is assumed that under acidic conditions ONOO^- is generated. Several recent studies have indicated that ONOO^- is one of the most potent oxidizing molecules in the field of biology and is a key agent in oxidative stress mechanisms. It is able to oxidize organic molecules directly or through H^+ or CO_2 -catalyzed homolysis (yielding NO_2 , OH , or carbonate anion radical $\text{CO}_3^{\cdot-}$). As for direct reactivity, it has affinity with key parts of proteins such as thiols, iron/sulfur centers, and zinc fingers. The lifetime of ONOO^- is relatively short, nonetheless, it can still cross cellular membranes and reach deep within the cell, enabling it to interact with most of the important biomolecules. Because of its instability, measuring the amount of ONOO^- produced is a considerable challenge.^{32,62,63}

To explain the significant difference in inactivation efficiency between spores exposed to plasma gas phase species and those exposed to PAB, the persistence of the fungal spores must be considered. These difference could be attributed to the surface characteristics of *Aspergillus* spp. spores which have extremely hydrophobic properties due to the presence of proteins known as hydrophobins. These proteins cause spores to clump together and form aggregates in aqueous solution by association of their hydrophobic molecules with each other rather than with other molecules within the liquid.⁶⁴ Hydrophobic properties of the surface of the spores in relation with their insufficient inactivation in water was already discussed in the study by Ouf et al., where double atmospheric pressure cold plasma was used with intention to inactivate different mold species during water washes of fruit.⁶⁵ Conversely, spores spread on glass and allowed to dry are not protected with liquid and are therefore far less likely to form such aggregates, and are therefore more vulnerable to the reactive plasma species. This is in agreement with the SEM images presented in Figure 5 and 7, where plasma treated spores display considerable damage after gas phase exposure, and little change after PAB exposure. It is well-known that O_3 and related ROS are important in terms of fungicidal activity of liquids.⁶⁶ In the case of this study, it was assumed that only a small amount of O_3 reached the aqueous phase due to its low Henry's law constant, and the number of potential loss pathways arising due to the presence of other plasma generated species, especially at a low pH. In all cases, the pH of the PAB was seen to drop and it is commonly accepted that the fungicidal effect in acidic conditions is less significant since many mold species tolerate low pH.⁶⁷ This is not the case when PAB is used as an antimicrobial agent, since many bacterial species are significantly less tolerant to a low pH

compared to their fungal counterparts. Nevertheless, a low pH can affect the electrostatic properties of the mold spores, resulting in an increase of zeta potential, meaning spores are likely to be more dispersed throughout the liquid volume, which can negatively affect the development of mold hyphae, mycelia and arrest mold growth.⁶⁸

Overall, it is clear that gas phase CAP treatment is an extremely effective and environmentally friendly method for fungal inactivation, which requires no consumables and produced no harmful residues. Based on the results presented, a greater understanding of CAP technology gas been uncovered and thus may accelerate the translation of this innovative green technology for the decontamination of food, living areas or use in other relevant environments. Conversely, the results with plasma treated liquid did not demonstrate promising results. The difference between surface and liquid inactivation efficacy was attributed to the hydrophobic surface properties of spores, which makes them considerably more resilient to RONS when in the liquid phase.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b05386.

Table S1. Comparison of nitrite and nitrate content between plasma activated deionized water (PAW) and plasma activated broth solution (PAB), using test strips. Figure S1. CAP effluent treatment of *A. flavus* spores spread on agar plates: (a), (b) and (c) log numbers of new grown spore units after low, medium and high power plasma treatment. A one way ANOVA was performed between treated samples and control samples (0 s). P values = 0.0332 (*); 0.0021 (**); 0.0002 (***) < 0.0001 (****). Figure S2. Liquid phase chemistry of PAB, obtained after the plasma treatment of 0.5 mL of broth solution; (a) pH values of PAB; (b) concentration of hydrogen peroxide; (c) concentration of nitrite; (d) concentration of nitrate. Figure S3. Liquid phase chemistry of PAB, obtained after the plasma treatment of 1.5 mL of broth solution; (a) pH values of PAB; (b) concentration of hydrogen peroxide; (c) concentration of nitrite; (d) concentration of nitrate (PDF)

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Table S1. Comparison of nitrite and nitrate content between plasma activated deionized water (PAW) and plasma activated broth solution (PAB), using test strips.

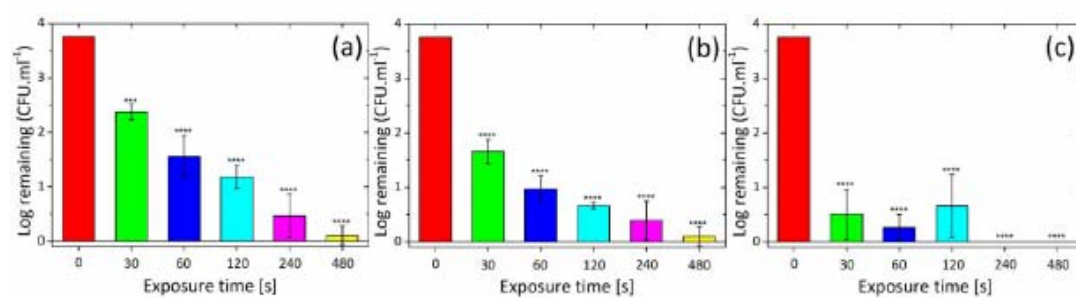
Figure S1. CAP effluent treatment of *A. flavus* spores spread on agar plates: (a), (b) and (c) log numbers of new grown spore units after low, medium and high power plasma treatment. A one way ANOVA was performed between treated samples and control samples (0 s). P values – 0,0332 (*); 0,0021 (**); 0,0002 (***); <0,0001 (****).

Figure S2. Liquid phase chemistry of PAB, obtained after the plasma treatment of 0.5 ml of broth solution; (a) pH values of PAB; (b) concentration of hydrogen peroxide; (c) concentration of nitrite; (d) concentration of nitrate.

Figure S3. Liquid phase chemistry of PAB, obtained after the plasma treatment of 1.5 ml of broth solution; (a) pH values of PAB; (b) concentration of hydrogen peroxide; (c) concentration of nitrite; (d) concentration of nitrate.

Table S1.

Concentration (mg/L)					
Low power		Medium power		High power	
PAW	PAB	PAW	PAB	PAW	PAB
10	10	10	10	0	0
10	10	10	10	10	10
10	10	10	10	25	25
25	25	25	25	50	50
25	25	100	100	100	100
50	50	250	250	250	250

**Figure S1.**

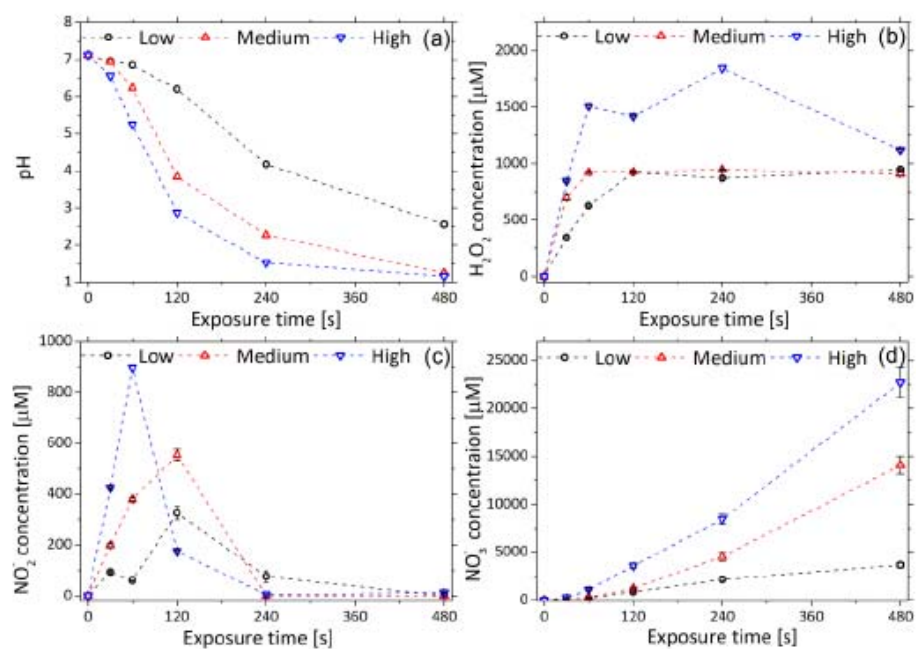


Figure S2.

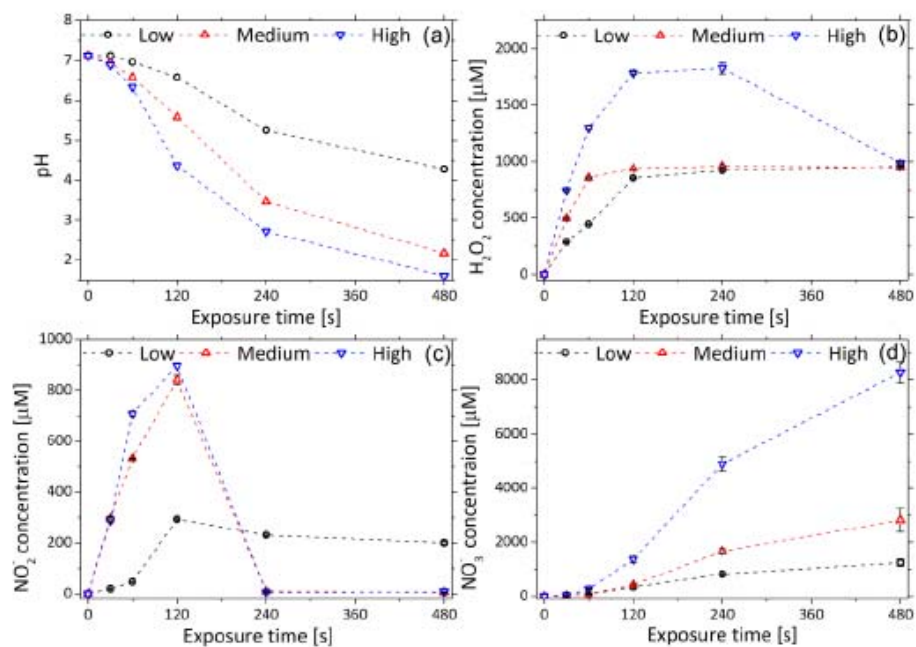


Figure S3.

Chapter 4

Mycotoxin Decontamination Efficacy of Atmospheric Pressure Air Plasma

This chapter explores the decontamination efficiency of the CAP regarding the different types of mycotoxins. The reported method is based on CAP created in the ambient air. To demonstrate our approach, the efficiency of the method was evaluated through comparison with conventional UV-C irradiation for the case of AFs, trichothecenes, FBs and ZEN.

CAP was proven to be more efficient, resulting in more than 90 % of decontamination of the sum of the mycotoxins. Moreover, mycotoxins such as AFG₁ and ZEN were completely removed. The differences in degradation rates may be attributed to the stability of the chemical structure of the particular mycotoxins. As demonstrated, trichothecenes were the most persistent mycotoxins. Nonetheless, CAP treatments reduced the concentrations of all mycotoxins under the maximum levels allowed in the selected food products.

This chapter addresses thesis Objectives 1.

Regarding my contribution: I performed the CAP and UV-C treatments of the mycotoxins. I jointly wrote the paper with co-authors.

*Communication*

Mycotoxin Decontamination Efficacy of Atmospheric Pressure Air Plasma

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Abstract: Mycotoxins, the toxic secondary metabolites of mould species, are a growing global concern, rendering almost 25% of all food produced unfit for human or animal consumption, thus placing immense pressure on the food supply chain. Cold Atmospheric pressure Plasma (CAP) represents a promising, low-cost, and environmentally friendly means to degrade mycotoxins with negligible effect on the quality of food products. Despite this promise, the study of CAP-mediated mycotoxin degradation has been limited to a small subset of the vast number of mycotoxins that plague the food supply chain. This study explores the degradation of aflatoxins, trichothecenes, fumonisins, and zearalenone using CAP generated in ambient air. CAP treatment was found to reduce aflatoxins by 93%, trichothecenes by 90%, fumonisins by 93%, and zearalenone by 100% after 8 minutes exposure. To demonstrate the potential of CAP-mediated mycotoxin degradation against more conventional methods, its efficiency was compared against ultraviolet C (UVC) light irradiation. In all cases, CAP was found to be considerably more efficient than UVC, with aflatoxin G₁ and zearalenone being completely degraded, levels that could not be achieved using UVC irradiation.

Keywords: mycotoxins; cold atmospheric pressure plasma; decontamination

Key Contribution: For the first time, this study explores the interaction of CAP with a number of different problem mycotoxins, highlighting impressive degradation efficiencies compared to more conventional UVC exposure.

1. Introduction

Mycotoxins are toxic compounds produced during the secondary metabolism of filamentous fungi and are known to regularly spoil food and feed products. Presently, more than 400 structurally different mycotoxins have been identified, including aflatoxins (AFs), ochratoxins, fumonisins (FBs), patulin, zearalenone (ZEN), ergot alkaloids, and trichothecenes, including deoxynivalenol (DON), HT-2 and T-2 toxin [1]. The recent and rapid increase in mycotoxin food contamination represents one of the main concerns in the field of agriculture and food production [2].

Most mycotoxins are chemically and thermally stable during food processing, meaning such contamination is extremely difficult to remove. They can harm human health through a wide range of toxic effects, including carcinogenicity, teratogenicity, hepatotoxicity, mutagenicity, neurotoxicity

effects, and the disruption of both immune and endocrine systems [3]. In essence, the food supply chain urgently needs a mycotoxin decontamination method that is low-cost, highly effective, and can be applied to contaminated food to minimise waste and enhance safety for the consumer. Current management strategies for controlling mycotoxin occurrence are mostly based on chemical, biological, and physical approaches [4]. Amid these, ultraviolet (UV) irradiation is one of the most frequently used non-thermal food processing methods [5]. Several studies have identified that UV irradiation is a potential technology for the decontamination of mycotoxins such as AFs, trichothecenes, and ZEN [6–9]. Nevertheless, degradation efficiencies can vary widely due to differences in irradiation conditions, meaning the exposure times that are necessary to achieve a satisfactory level of decontamination are often too long to be relevant for large-scale food production.

Recently, exciting preliminary results have shown that Cold Atmospheric pressure Plasma (CAP) has considerable potential to reduce food contamination and improve food safety [10–12]. Plasma is the fourth state of matter or quasi-neutral ionized gas, primarily composed of photons, ions, free electrons, as well as atoms in their ground or excited states with a net neutral charge. The strong electric fields used in generating cold plasma accelerate electrons to energies capable of ionizing, electronically exciting, dissociating, and heating the constituents of the background gas. In air, this results in over 50 distinct chemical species, including the ionic species of H^+ , H^- , O^+ , O^- , H_3O^+ , OH^- , and N_2^+ ; the excited and ground states of N_2 , O , NO , and OH ; and longer-lived species such as O_3 , NO_2 , and N_2O ; collectively, these are referred to as reactive oxygen and nitrogen species (RONS) [13]. The concept of treating food with non-thermal plasma for the purposes of microbial inactivation has been under consideration for well over a decade [14,15], and it has been shown that the RONS produced within plasma are highly effective antimicrobial agents that are also capable of degrading a wide variety of toxic compounds, including mycotoxins [16–18].

In this study, the efficiency of a scalable and low-cost CAP-based approach was compared with ultraviolet C (UVC) irradiation for the degradation of a diverse array of mycotoxins. Aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2), DON, 3-Acetyl DON (3-AcDON), 15-Acetyl DON (15-AcDON), diacetoxyscirpenol (DAS), HT-2, and T-2 were considered, as well as fumonisins B_1 (FB_1), B_2 (FB_2), and ZEN. The CAP system was based on a surface barrier discharge (SBD) configuration, using ambient air as the working gas and two different powers, leading to two different CAP chemistries [19,20]. To determine decontamination efficacy, the remaining mycotoxin concentration following CAP or UVC exposure was assessed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

2. Results and Discussion

The presented study exposes the high decontamination potential of CAP against mycotoxins, which was especially evident when compared to the commonly used UVC irradiation. The efficiency of CAP mycotoxin removal was evaluated according to the current European Union (EU) legislation dictating the maximum permissible levels of mycotoxin residual on food products. Strict monitoring procedures are currently mandated across the EU for the mycotoxins AFB_1 , DON, OTA, and ZEN as well as the total combined amount of AFs, HT-2, and T-2 and FBs [21–23]. The maximum levels allowed in selected food products are listed in Table 1.

It is widely reported that AF are the most toxic mycotoxins, with a chemical structure that categorises them as members of the difuranocoumarins. Critically, AFB_1 has been identified as one of the leading causes for liver cancer worldwide as a result of toxic effects such as mutagenicity and cytotoxicity [24]. For the experiments detailed here, AFs were deposited on clean glass coverslips with the following concentrations: 18 $\mu\text{g/kg}$ of AFB_1 and AFG_1 , and 4 $\mu\text{g/kg}$ of AFB_2 and AFG_2 . As shown in Figure 1, following CAP exposure, the concentrations of AFB_1 were reduced to values lower than 0.006 $\mu\text{g/kg}$ after only 30 s of exposure (Figure 1a). The most efficient CAP condition was found to be the low power, reducing AFB_1 to 0.003 $\mu\text{g/kg}$ (>99.99% decontamination). In both cases, the AFB_1 concentration was reduced well below the maximum level allowed according to EU

legislation (2 µg/kg). The performance of CAP decontamination was even higher in the case of AFG₁ (Figure 1c), where complete decontamination was noted after 60 s of treatment with the high-power operated CAP.

Table 1. Maximum levels of mycotoxins allowed in selected food products in the European Union (EU) [21–23].

Mycotoxin	Maximum Level Allowed [µg/kg]	Type of Food Product
AFB ₁ /AFs	2/4	All cereals and all products derived from cereals, including processed cereal products, with the exception of maize; processed cereal-based foods and baby foods for infants and young children; and dietary foods for special medical purposes, intended specifically for infants
DON	750	Cereals intended for direct human consumption; cereal flour (including maize flour, maize meal, and maize grits); and bran as an end product marketed for direct human consumption and germ, with the exception of processed cereal-based foods and baby foods for infants and young children
HT-2 & T-2	200	Oats for direct human consumption
	2000	Oat milling products for feed (husks)
FBs	1000	Maize flour, maize meal, maize grits, maize germ, and refined maize oil
ZEN	200	Maize intended for direct human consumption, maize flour, maize meal, maize grits, maize germ, and refined maize oil

In contrast, Figure 1b,d reveals that AFB₂ and AFG₂ were more persistent against both decontamination approaches used in the study. Nevertheless, high-power CAP conditions led to the highest level of degradation, reducing AFB₂ by 70% and AFG₂ by 74%. Despite the incomplete degradation, the sum of AFs exposed to CAP for 480 s did not exceed the maximum level allowed in cereals, i.e., 4 µg/kg (Figure 2). UVC irradiation, on the other hand, initially induced a rapid decrease in AFs concentration over the first few seconds of treatment, after which no further degradation was observed, yielding a total reduction of less than 50%.

In contrast to AFs, trichothecenes are known to be particularly resistant to many decontamination approaches. They represent a large group of compounds with approximately 170 different types of metabolites with a common a tetracyclic-12,13-epoxy skeleton. Their toxicity is based on their ability to interfere with the synthesis of proteins, resulting in acute poisoning symptoms such as diarrhoea, vomiting, and nausea [25–27]. Notably, Figure 3 indicates that a significantly higher degradation efficiency was achieved following CAP treatments compared to UV irradiation. In the case of DON, the greatest reduction was achieved after 480 s of CAP treatment under high power conditions, with just 0.11 mg/kg of DON remaining from a starting concentration of 2.7 mg/kg (Figure 3a), which falls below the maximum allowable level for cereals (0.75 mg/kg). Low power plasma conditions did not reach a comparable decontamination efficiency; nevertheless, the degradation still exceeded that found with UVC irradiation. Furthermore, comparable decontamination trends were also observed in the case of 3-AcDON, 15-AcDON, HT-2, and T-2 (Figure 3b,c,e,f). Their initial concentration was 2.5 mg/kg with the exception of HT-2, which had the higher concentration of 3.5 mg/kg. A significant amount, 0.5 mg/kg, of both 15-AcDON and T-2 was detected following even the longest exposures CAP, indicating that they are some of the most persistent trichothecenes to CAP-based approaches. Furthermore, none of the approaches examined in this study were able to reduce the sum of HT-2 and

T-2 under the maximum levels allowed in food products such as processed oats intended for direct human consumption (Figure 4). Nonetheless, CAP treatments reduced the amount of HT-2 and T-2 under the 2 mg/kg, which is the maximum permissible level allowed in oat milling products used for feed [23]. Among trichothecenes, DAS was the most sensitive to CAP (Figure 3d), reaching a 97% reduction after a 480 s treatment under high power CAP conditions.

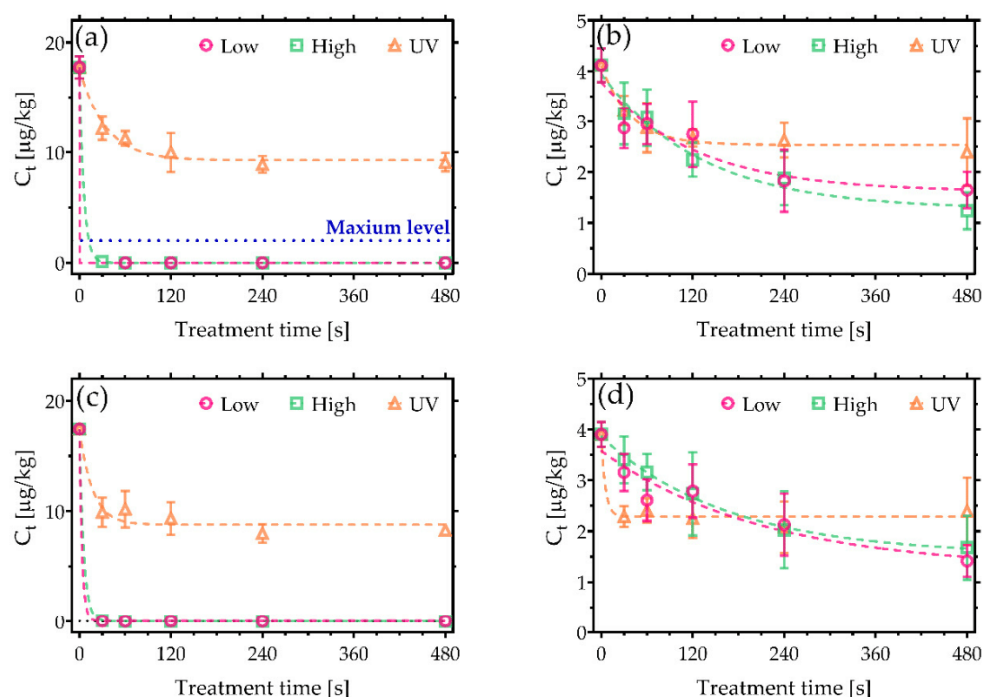


Figure 1. Cold Atmospheric pressure Plasma (CAP) decontamination of aflatoxins (AFs) under low and high discharge power conditions and a comparison against UVC treatment. Plots show the reduction in concentration of (a) AFB₁, (b) AFB₂, (c) AFG₁, and (d) AFG₂. The maximum permissible level of AFB₁ allowed in cereals of 2 $\mu\text{g/kg}$ is shown in (a) [21].

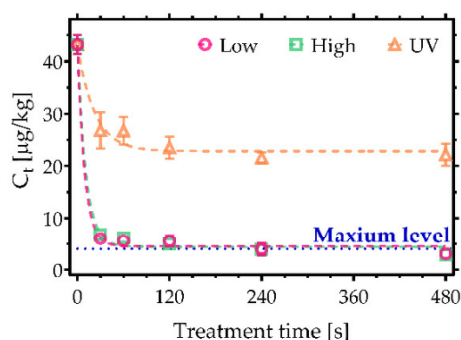


Figure 2. CAP decontamination of AFs considering the sum of their concentrations based on the air surface barrier discharge (SBD) plasma system operated at low and high discharge power. A comparison with UVC irradiation. Maximum level of AFs sum allowed in cereals is 4 $\mu\text{g/kg}$ [21].

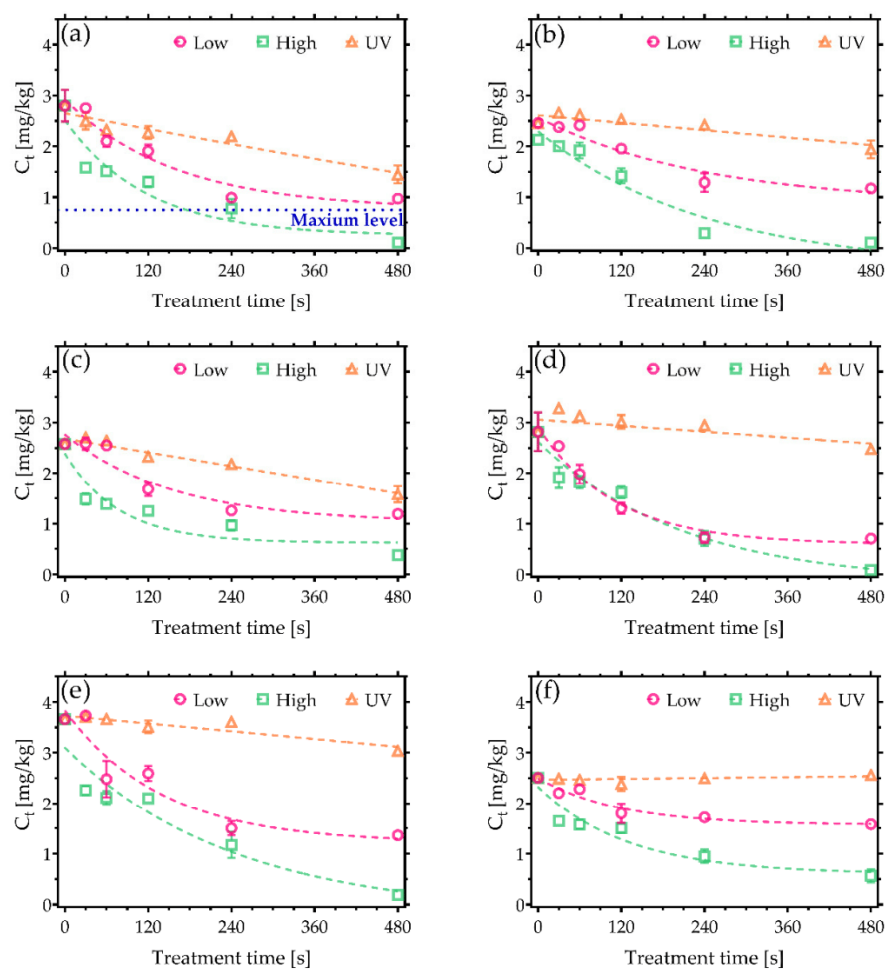


Figure 3. CAP decontamination of trichothecenes under low and high discharge power conditions and a comparison against UVC treatment. Plots show the reduction in concentration of (a) deoxynivalenol (DON), (b) 3-Acetyl DON (3-AcDON), (c) 15-Acetyl DON 15-AcDON, (d) diacetoxyscirpenol (DAS), (e) HT-2, and (f) T-2 as a function of CAP exposure time. The maximum permissible level of DON allowed in cereals of 0.75 mg/kg is shown on (a) [22].

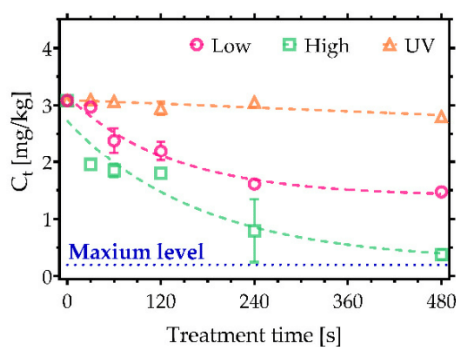


Figure 4. CAP decontamination of HT-2 and T-2 considering the sum of their concentrations based on the air surface barrier discharge (SBD) plasma system operated at low and high discharge power. A comparison with UVC irradiation. Maximum level of HT-2 and T-2 allowed in oats is 0.2 mg/kg [23].

FBs are polyketides with characteristic 2-propane-1,2,3-tricarboxylic acids esterified to an aminopolyol chain. Through their strong resemblance to sphinganine, they can disrupt cell functions, which involve sphingolipids, including cell proliferation, cell differentiation, and apoptosis. Belonging to a class of non-genotoxic carcinogens, they contribute to both liver and kidney cancers [28]. In this study, the starting concentrations of FBs were 2.7 mg/kg for FB₁ and 2.9 mg/kg for FB₂. In both CAP cases, a degradation in excess of 90% was observed (Figure 5), significantly higher than that achievable with UVC exposure.

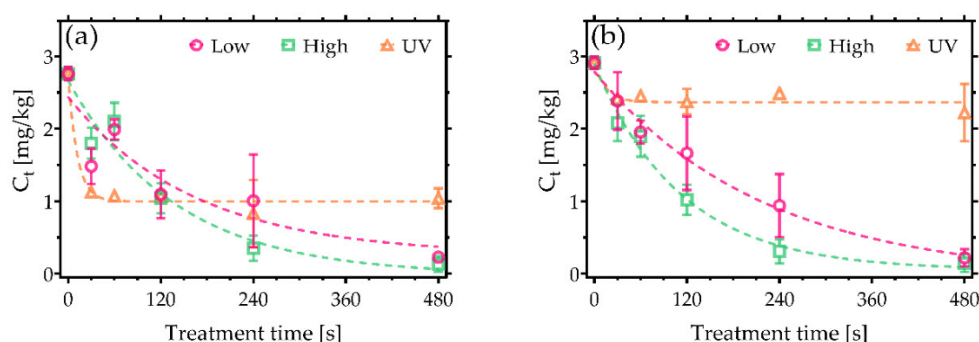


Figure 5. CAP decontamination of FBs under low and high discharge power conditions and a comparison against UVC treatment. Plots show the reduction in concentration of (a) FB₁ and (b) FB₂ as a function of exposure time.

Figure 6 shows the sum of both FBs. Both CAP conditions reduced the summed concentration below the maximum permissible level for maize products, 1 mg/kg [22].

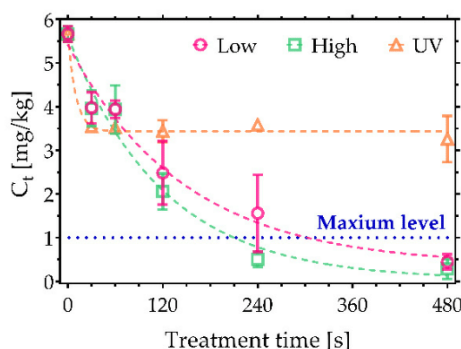


Figure 6. CAP decontamination of FBs considering the sum of their concentrations based on the air SBD plasma system operated at low and high discharge power. A comparison with UVC irradiation. Maximum level of FBs sum allowed in maize products is 1 mg/kg [22].

Finally, ZEN was considered: it has a 6-(10-Hydroxy-6-oxo-trans-1-undecenyl)- β -resorcylic acid lactone structure that has shares similarities with the human sex hormone 17 β -estradiol. Consequently, it can bind to oestrogen receptors, leading to endocrine disruption and reproduction issues [29]. In this study, an initial ZEN concentration of 2.5 mg/kg was used. Complete removal was observed following exposure to CAP after 60 s of treatment under high power conditions (Figure 7). Notably, only 45% degradation was observed after 480 s of UVC irradiation. Moreover, UVC irradiation was found to induce the formation of an additional chromatogram peak near the primary peak, likely resulting from photoisomeration (Figure S1). It is well known that ZEN exists in two stereoisomeric forms; in the presence of UV photons, the naturally occurring ZEN trans form can be converted to the more stable cis-ZEN [30]. In contrast, no cis-ZEN was observed after the treatment with CAP.

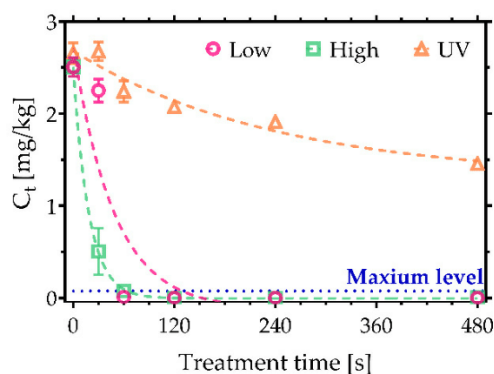


Figure 7. Decontamination of zearalenone (ZEN) based on the air SBD plasma system operated at low and high discharge power. A comparison with UVC irradiation. Maximum level of ZEN allowed in cereals is 0.1 mg/kg [22].

A limited number of previous CAP studies have considered the decontamination of different kinds of mycotoxins. Given the highest toxicity among mycotoxins, majority of studies have explored the degradation of AFs [16,17,31]. A dielectric barrier discharge (DBD) system (AcXys Technologies, St. Martin Le Vinoux, France) was used for AFs removal with an N_2 as carrier gas, resulting in 100% decontamination of AFB_1 after 4 min of treatment [17]. As in our case, AFB_1 and AFG_1 were more sensitive to CAP compared to AFB_2 and AFG_2 . Moreover, studies using UV irradiation as an AFs decontamination approach showed similarities with the findings reported in this study, demonstrating that significant levels of decontamination can only be reached with long exposures (>30 min) [6,8,32]. Apart from AFs, a study by ten Bosch et al. explored the efficiency of air-based DBD plasma in decontamination of various mycotoxins, including DON, T-2, FB_1 , and ZEN [18]. Compared to our results, the degradation rates varied with the type and structure of mycotoxin.

To understand the decontamination based on CAP, the physical-chemical properties of CAP must be considered. CAP-induced chemical degradation is mostly initiated through the generation of the variety of RONS. Short-lived species such as atomic oxygen (O), hydroxyl radical (OH), atomic nitrogen (N), and superoxide ion (O_2^{-1}) are produced next to the plasma region. Following this, they are distributed away from the electrode, where they react with each other, forming long-lived species such as ozone (O_3), hydrogen peroxide (H_2O_2), nitric oxide (NO), and other nitrogen oxides (NO_x) [19]. Experiments done in this study were performed with the 5 mm distance between the mycotoxin sample and outer electrode, meaning that long-lived species contributed the most to the CAP degradation of mycotoxins.

Low power conditions induce the formation of reactive oxygen species (ROS)-dominant plasma effluent chemistry with O_3 as the primary long-lived element. On contrary, a significant rise of the reactive nitrogen species (RNS) is observed under high power conditions, including N_2O and NO_2 , eventually resulting in O_3 decomposition. Alternatively, plasma effluent can be also defined as chemistries containing low and high dose of RONS, corresponding to the low and high power conditions [19,20,33]. The interaction between aqueous interface of wet mycotoxin sample and long-lived RONS induces the formation of a special liquid chemistry, which reintroduces very reactive, indirectly produced short-lived species, including OH and peroxynitrite ($ONOO^-$) [34,35].

CAP RONS chemical degradation mostly involves radical reactions. RONS have a tendency to react with the least stable sites of the molecules such as double bonds between two carbon atoms, especially with those that are geometrically more exposed. RONS double bond attack is carried out through different mechanisms, starting with hydrogen abstraction, epoxidation, and oxidation of the carbon atoms with bond break as another possible result [36,37]. Such double bonds are included in the chemical structures of AFB_1 , AFG_1 , and ZEN, which were the most sensitive to the CAP treatments. In contrast, the rest of the mycotoxins possess more stable structures and are therefore more resistant

to the CAP RONS. In addition, samples with trichothecenes contained a significantly higher number of mycotoxin molecules compared to other mycotoxins, meaning that a larger number of RONS is necessary for their complete removal. Consequently, longer exposure times are required for higher decontamination rates.

3. Conclusions

Mycotoxin members AFs, trichothecenes, FBs, and ZEN were successfully decontaminated after exposure to the CAP generated RONS. CAP based on the highly scalable air SBD system is known to produce high concentrations of RONS already in short operating times, which can react with a large number of mycotoxin molecules at once, leading to considerably higher decontamination efficiency compared to UVC irradiation, which is one of the most frequently used food processing approaches. Regarding their short lifetime, RONS decompose shortly after their formation, leaving no residuals.

4. Materials and Methods

4.1. Preparation of Mycotoxins and CAP Treatments

For the treatments, stock solutions of mycotoxins were prepared from their standards purchased from Romer (Union, MO, USA) in the mixture of acetonitrile and deionized water (70% acetonitrile/30% water (*v/v*)) in order to obtain better decontamination performance. The concentrations of mycotoxin stock solutions are listed in the Table 2. A volume of 50 μ L of each mycotoxin solution was deposited on a glass coverslip and left for 5 minutes to dry. Immediately after, the glass coverslips with a layer of mycotoxin were exposed to CAP.

Table 2. The concentrations of the stock solutions of mycotoxins used in presented study.

Mycotoxin	Stock Solution Concentration	Acetonitrile/diH ₂ O Ratio
AFs		
AFB ₁ , AFG ₁	0.2 μ g/mL	2:1
AFB ₂ , AFG ₂	0.05 μ g/mL	
Trichothecenes		
DON, 3-AcDON, 15-AcDON, DAS, HT-2, T2	27 μ g/mL	2:1
FBs		
FB ₁ , FB ₂	25 μ g/mL	1:1
ZEN	27 μ g/mL	2:1

The CAP treatments were performed with highly scalable air SBD system similar to that reported by Modic et al. [14]. The plasma was generated from specially fabricated electrode unit made of 1 mm thick quartz disc placed between two electrodes and plastic housing. The inner copper electrode was connected with high voltage source, covered with epoxy resin to prevent the formation of plasma. On the other side of the quartz disc, the outer aluminium electrode in the shape of a mesh was placed and connected to the power source ground. After the application of a high voltage AC signal, plasma was generated at the edges of aluminium mesh. The electrode unit was attached to the homemade power source, which operated at voltages 7 to 10 kV peak to peak at frequency of 40 kHz.

Two plasma powers were used, termed low and high power, to generate contrasting plasma chemistries ranging from an ROS dominated mode (at low power) and an RNS dominated mode (at high power). A full description of the RONS produced under different operating power regimes can be found in our previous work [19,20,33].

During the treatments, the electrode unit was placed over the glass coverslips at fixed distance 5 mm between plasma ignition point and mycotoxin sample. The mycotoxins were treated for different time durations (30, 60, 120, 240, and 480 s). Following the exposures, the mycotoxins were washed

from the surface of the glass coverslips with 0.5 mL of acetonitrile, and the solutions were transferred to vials for further LC-MS/MS analysis. For the comparison, mycotoxins were also irradiated with UVC lamp, considering equal treatment distance and exposure times. UVC irradiation was performed with standard UVC germicidal lamp (Philips, Amsterdam, The Netherlands), with highest intensity line of Hg at 253.65 nm (Figure S2).

The experiments were performed in multiple repetitions to provide statistical significance, which was analysed with one-way ANOVA and nonlinear curve fit function (GraphPad Prism 8, GraphPad, San Diego, CA, USA).

4.2. LC-MS/MS Analysis of Treated Mycotoxins

Mycotoxins in solutions were quantified with LC-MS/MS (Waters, USA). All chromatographic separations were performed on a Zorbax Eclipse Plus C18 Rapid Resolution HD column, 2.1 × 100 mm, 1.8 µm (Agilent Technologies, Santa Clara, CA, USA). The chromatographic separation was performed by mixing of two mobile phase components (A and B) in isocratic mode (AFs) or gradient mode (trichothecenes, FBs, and ZEN). Component A was deionized water and component B was methanol, both containing 0.5% acetic acid and 2.5 mM ammonium acetate. Sample injection volume was 10 µL.

Isocratic elution of AFs was performed with a mixture of component A and B in the ratio 60:40. Sample was injected into column with mobile phase flow rate 0.3 mL/min and temperature 40 °C. Detection with MRM (multiple reaction monitoring) mode and ESI in positive ionization mode was performed in following conditions: source temperature 150 °C, desolvation temperature 200 °C, the capillary voltage 3.7 kV, the cone gas flow 20 L/h, and desolvation gas flow 800 L/h. For measuring the presence of AFB₁, AFB₂, AFG₁, and AFG₂, the ions at *m/z* 313, 315, 329, and 331 were monitored, respectively.

For the separation of other mycotoxins, time-programmed gradient was applied with initial composition 95% of A and 5% of B. The portion of component B was linearly increased to 40% within 4 min and further increased to 70% within the next 8 min. This latter composition was held for 4 min, and then component B was increased to 90% in 1.5 min. The proportion of component B was held at 90% for 2.5 min and then returned back to 5% in 1 min. The final composition was held for 4 min. The mobile phase flow rate was 0.3 mL/min, and the column temperature was 40 °C. MS/MS analysis was carried out in MRM mode. The mass spectrometer was operated in the electrospray ionization mode (ESI). During the run, ESI source was switching between positive (ESI+) and negative (ESI−) mode. Capillary voltage in ESI+ mode was 3.4 kV and in ESI− mode 3.0 kV. The desolvation temperature was 500 °C, the ion source temperature was 150 °C, and the collision cell voltage and cone voltages were optimized for each mycotoxin as shown in Table 3.

Table 3. Tandem Mass Spectrometry (LC-MS/MS) detector parameters.

Mycotoxin	Ionization Mode	RT (min)	Precursor Ion (m/z)	Quantifier Ion (m/z)	Qualifier Ion (m/z)	Cone Voltage (V)	Collision Energy (V)
DON	ESI+	3.14	297.3	203.1	249.1	18/18	16/10
3-AcDON	ESI+	5.00	339.1	203.1	137.0	24/14	16/5
15-AcDON	ESI+	5.00	339.1	136.95	261.1	16/16	12/10
DAS	ESI+	7.06	384.3	307.2	247.2	20/20	14/10
HT-2	ESI+	8.91	442.4	215.1	263.2	12/12	16/14
T-2	ESI+	10.27	484.4	185.1	215.2	18/18	24/20
ZEN	ESI−	11.30	317.20	131.0	174.95	35/40	22/24
FB ₁	ESI+	10.20	722.4	334.2	352.2	30/30	40/35
FB ₂	ESI+	12.70	706.4	318.2	336.2	35/35	40/40
OTA	ESI+	11.10	404.2	221.0	239.0	24/24	36/24

The areas of the chromatogram peak of corresponding mycotoxin were measured, out of which the concentrations were calculated, using the standard curve obtained from the serial dilutions of standards. Considering the extraction of mycotoxins from food matrices, the concentrations were then converted from $\mu\text{g/mL}$ (ppm) to $\mu\text{g/kg}$ or mg/kg .

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6651/11/4/219/s1>, Figure S1: Chromatogram of ZEN obtained after the exposure to UVC irradiation; Figure S2: Hg intensity lines of UVC germicidal lamp.

Author Contributions: N.H. designed and performed the experiments. She also wrote the manuscript. M.M. helped with design of the experiments and provided knowledge from the use of plasma for the biological purposes. J.L.W. designed and provided plasma system and helped with the knowledge from plasma technology. G.T.-K. and J.B. performed LC-MS analysis and helped with the interpretation of the results and with the experience in the field of mycotoxins and food safety. U.C. managed the process of the making of the manuscript and conceived the experiments. He helped with knowledge from the plasma technology point of view.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials: Mycotoxin Decontamination Efficacy of Atmospheric Pressure Air Plasma

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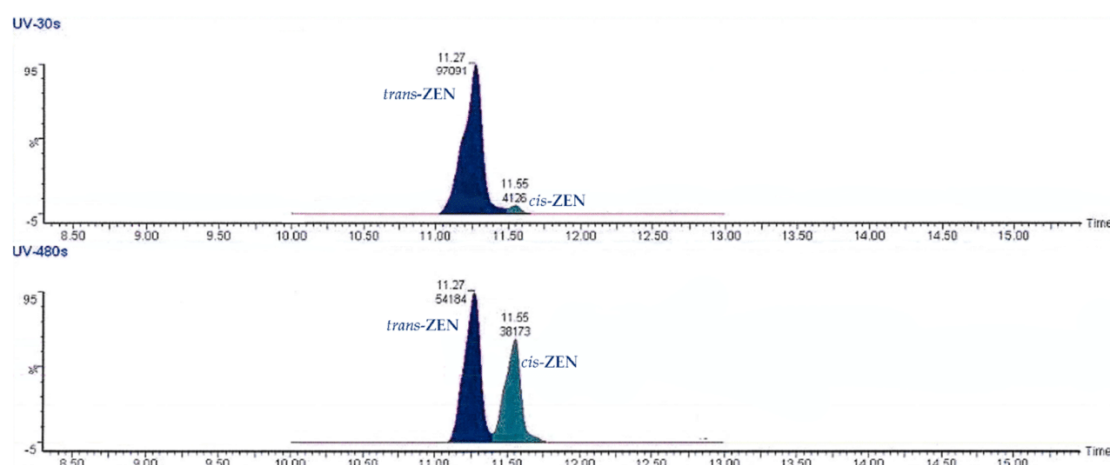


Figure 1. Chromatogram of ZEN obtained after the exposure to UVC irradiation.

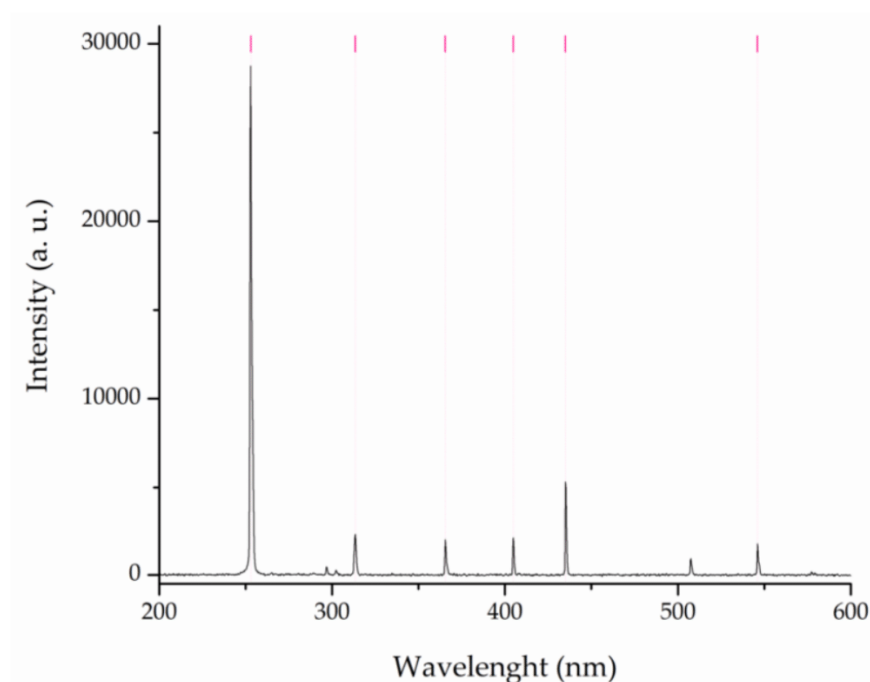


Figure 2. Hg intensity lines of UVC germicidal lamp.

Chapter 5

Reactive Oxygen and Nitrogen Species for Targeted Reduction of Mycotoxin Toxicity

Chapter 5 provides a case study based on a deterministic approach, where the degradation mechanisms are precisely explored, obtained from an air SBD plasma treatment of AFB₁.

Mycotoxin toxicity arises from specific chemical sites in the molecule, and several chemical methods have been developed to neutralise toxicity through the elimination of these molecular parts or complete degradation of the molecule, yet no study so far has provided a reliable investigation based on determination of mycotoxin decontamination mechanisms. This work reported the complete decontamination of AFB₁, which was not observed in the case of UV-C irradiation. It also addressed the application of a controlled dose of RONS to target the toxic sites of mycotoxin molecules. For the first time, the complete chemical pathways and molecular degradation mechanisms were revealed. We demonstrate that rapid scission of the vinyl bond between 8- and 9-position on the terminal furan ring of AFB₁ resulted in expeditious suppression of the molecules' toxic potential. Therefore, proper utilisation of RONS can open completely new channels for targeted molecule degradation processes in chemistry.

The supplementary information of the paper also provides major results based on the decontamination of AFB₁-contaminated corn. As observed, comparable decontamination efficiencies were achieved with no CAP-induced modifications of corn kernel surface detected.

This chapter addresses all thesis Objectives.

Regarding my contribution: I performed the CAP treatments of the AFB₁ and FTIR analysis of the treated AFB₁ and corn kernel surface. I jointly performed HRMS analysis and toxicological tests. I wrote the paper together with co-authors.

1 **Reactive oxygen and nitrogen species for targeted reduction of mycotoxin toxicity**

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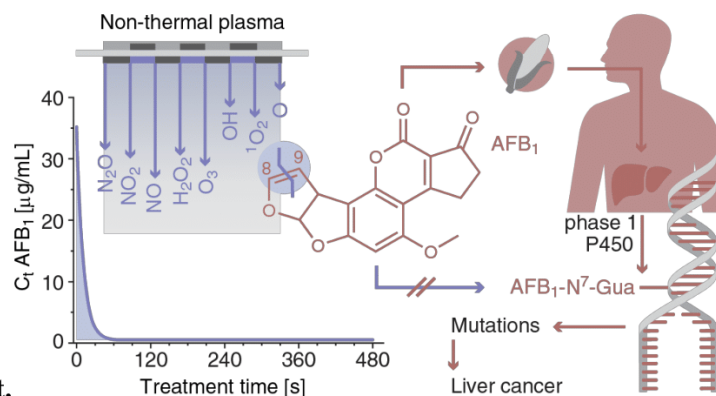
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Abstract. Mycotoxins are the secondary metabolites produced by filamentous fungi that are found on over 25 % of global food and feed crop. These highly toxic compounds are extremely difficult to eradicate meaning contaminated produce must be removed from the food chain in order to ensure public safety. Although numerous strategies have been proposed to remove mycotoxins from food products, no technique has proven sufficiently effective to protect consumers. Here we report an alternative and easily implemented approach using non-equilibrium plasma as a source of reactive oxygen and nitrogen species for targeted degradation of the potent aflatoxin B₁. We identify rapid scission of the vinyl bond between 8- and 9-position on the terminal furan ring of aflatoxin B₁ as being of paramount importance for the suppression of toxic potential. The reactive species mediated degradation pathways are elucidated, and it is demonstrated that the approach not only renders aflatoxin B₁ harmless but does so in an order of magnitude less time than other methods currently under investigation.



Graphical abstract.

Naturally occurring mycotoxins are stable toxic metabolites produced by various filamentous fungi species, which regularly cause the contamination of food crops such as cereals and nuts.¹ Currently, more than 400 structurally different mycotoxins have been identified, which can produce an assortment of toxic effects. Amongst these, aflatoxins are considered the greatest threat to human health, especially aflatoxin B₁ (AFB₁) to which more than half of the world's population is chronically exposed. AFB₁ is one of the most potent natural group 1 carcinogens, responsible for up to 155,000 (28 %) annual cases of human hepatocellular carcinoma cancer.^{2,3} For this reason, mycotoxins have become one of the most critical dietary risk factors, with the situation being further exasperated by the alarming increase in their occurrence on widely consumed food stuffs.⁴ Mycotoxin contamination is considered an unavoidable and unpredictable problem as they are readily found on crops where good agricultural, storage and processing practices have been implemented.⁵⁻⁸ To combat this growing issue, food producers are urgently seeking novel non-thermal decontamination methods that are able to rapidly eliminate mycotoxin contamination with negligible impact on the treated food product and environment. Current non-thermal approaches include UV irradiation, pulsed light treatment, gamma irradiation and ozonation, which induce the breakdown of the mycotoxin molecule by the absorption of high energies or interaction with highly reactive chemical species.^{9,10}

Recently, an innovative approach based on non-equilibrium gas plasma has emerged as an alternative and highly effective decontamination technology. The potential of cold atmospheric pressure plasma (CAP) to degrade persistent chemical contamination has been well known for several decades.^{11,12} However, recent advances in our ability to generate and sustain CAP in the open air have led to the development of plasma systems that no longer require the use of expensive vacuum equipment or noble carrier gases. The non-equilibrium nature of the discharge gives rise to electrons with energies exceeding the ionisation potential

of nitrogen and oxygen, yet heavy species, such as ions and neutrals, remain close to room temperature thus giving the discharge its non-thermal characteristics. The generation of non-equilibrium atmospheric pressure plasma in ambient air yields a vast array of charged and neutral species, including electrons, ions, ultraviolet photons and a wealth of highly reactive oxygen and nitrogen species (RONS) such as atomic oxygen (O), hydroxyl radical (OH), atomic nitrogen (N), superoxide (O_2^-), ozone (O_3), hydrogen peroxide (H_2O_2), nitric oxide (NO), and other nitrogen oxides (N_xO_y).¹³ It is the simultaneous generation of these RONS and other physicochemical phenomenon that lead to the oxidative nature of air plasma and are closely linked to their ability to degrade complex chemical molecules.¹¹ Given the obvious potential of CAP as a non-thermal decontamination technique, the CAP mediated degradation of several mycotoxins such as aflatoxin B₁ (AFB₁), deoxynivalenol (DON), zearalenone (ZEN), enniatins, fumonisin B₁ (FB₁), T2 toxin, sterigmatocystin and AAL toxin have recently been explored.^{14,15} These preliminary studies have provided an intriguing demonstration of the potential of CAP to remove mycotoxin contamination in comparison to other non-thermal decontamination methods that are currently being explored.¹⁶ Despite these encouraging preliminary results, the research performed to date does not provide insight into the underpinning plasma mediated degradation pathways and, perhaps more importantly, the identification of breakdown products and their potential toxicity remains unexplored.

Here we report a deterministic approach that features a model system for the degradation of mycotoxins with CAP produced RONS based on the AFB₁ molecule, which could be extended to other toxins. We propose a detailed degradation pathway of AFB₁ induced by CAP RONS with the identification of the most important degradation products and assessment of their toxicity using high-resolution mass spectroscopy (HRMS) and nuclear magnetic resonance spectrometry (¹H-NMR, 2D NMR) combined with toxicity studies performed following different levels of CAP exposure. The work offers a detailed insight into

conditions comparable to those of the CAP exposures, only 36% of the initial concentration of AFB₁ was degraded after 480 s of treatment (Fig. 1b).

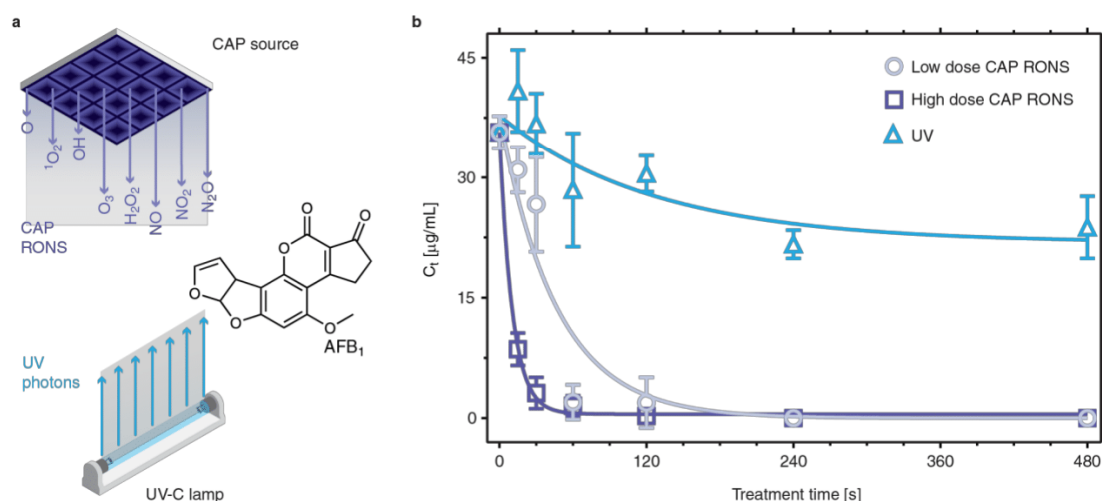


Figure 1 | Decontamination of AFB₁ with CAP RONS and UV-C irradiation. **a** schematic showing interaction between CAP RONS / UV-C photons and AFB₁ molecule; **b** reduction in the concentration of AFB₁ after treatment with CAP RONS and UV-C photons for different exposure times.

Assessment of degradation products using UPLC-QqTOF MS analysis. Beyond the quantification of AFB₁ degradation efficacy, UPLC-QqTOF MS was employed to characterise the degradation products resulting from CAP exposure. By application of tandem mass (MS/MS) fragmentation and accurate mass measurement, performed with QqTOF MS coupled with UPLC for superior separation and speed performance, a large number of degradation products were detected. Amongst these, the four most prevalent products are outlined in Fig. 2a. They appeared in both RONS treatment regimes, indicating that the different discharge power conditions used did not significantly alter the formation of different types of degradation products. It is clear that short exposures induced an increase in the concentrations of degradation products, followed by their progressive decline with prolonged exposure (Fig.

2b,c). A high dose of CAP generated RONS led to a rapid transition of the parent molecule to undetectable levels of breakdown products, whereas a low dose of CAP generated RONS yielded a moderate and traceable degradation process, providing ideal conditions to explore the degradation mechanisms. Consequently, further detailed analysis of AFB₁ degradation was performed using only low dose RONS conditions.

From fig. 2a it is clear that the parent molecule of AFB₁ eluted at a retention time of approximately 4.0 min with a protonated molecular ion $[M+H]^+$ at m/z 313 (C₁₇H₁₃O₆). The MS/MS fragmentation of AFB₁ consisted of gradual cleavages of carbonyl groups, categorised in three main steps: (1) double cleavage of carbonyl groups with an initial intermediate removal of methyl, resulting in fragment ions at m/z 285 and 257; (2) the removal of carbonyl with initial loss of methane; and (3) the cleavage of a carbonyl group, complementary to the cleavage presented in the first step (see Supplementary Fig. S3).

RONS exposure of AFB₁ initially induced the formation of product P₁, which exhibited a protonated molecule $[M+H]^+$ - 330 m/z (C₁₇H₁₅O₇) at the retention time of 3.3 min. P₁ was detected in notable quantities when AFB₁ was exposed to a low dose of CAP RONS between treatment times of 30 s and 120 s. In contrast, the highest P₁ concentration was observed after 15 s of treatment with a high dose of CAP RONS, ultimately dropping below the detection limit following 60 s of exposure. The molecular mass of P₁ is, in comparison to AFB₁, higher for a mass equivalent to one molecule of water, which also corresponded to the first fragment ion in the MS/MS spectrum at m/z 313 (see Supplementary Fig. S4). Additionally, a further MS/MS fragmentation pattern corresponded to the one observed in the MS/MS spectrum of AFB₁, indicating that P₁ retained an identical structural frame.

The most abundant degradation product, labelled P₂, began to develop shortly after the appearance of P₁, at a retention time of 3.7 min. It matched the protonated molecular ion $[M+H]^+$ - 299 m/z (C₁₆H₁₁O₆), which reached a maximum concentration after 60 s of exposure

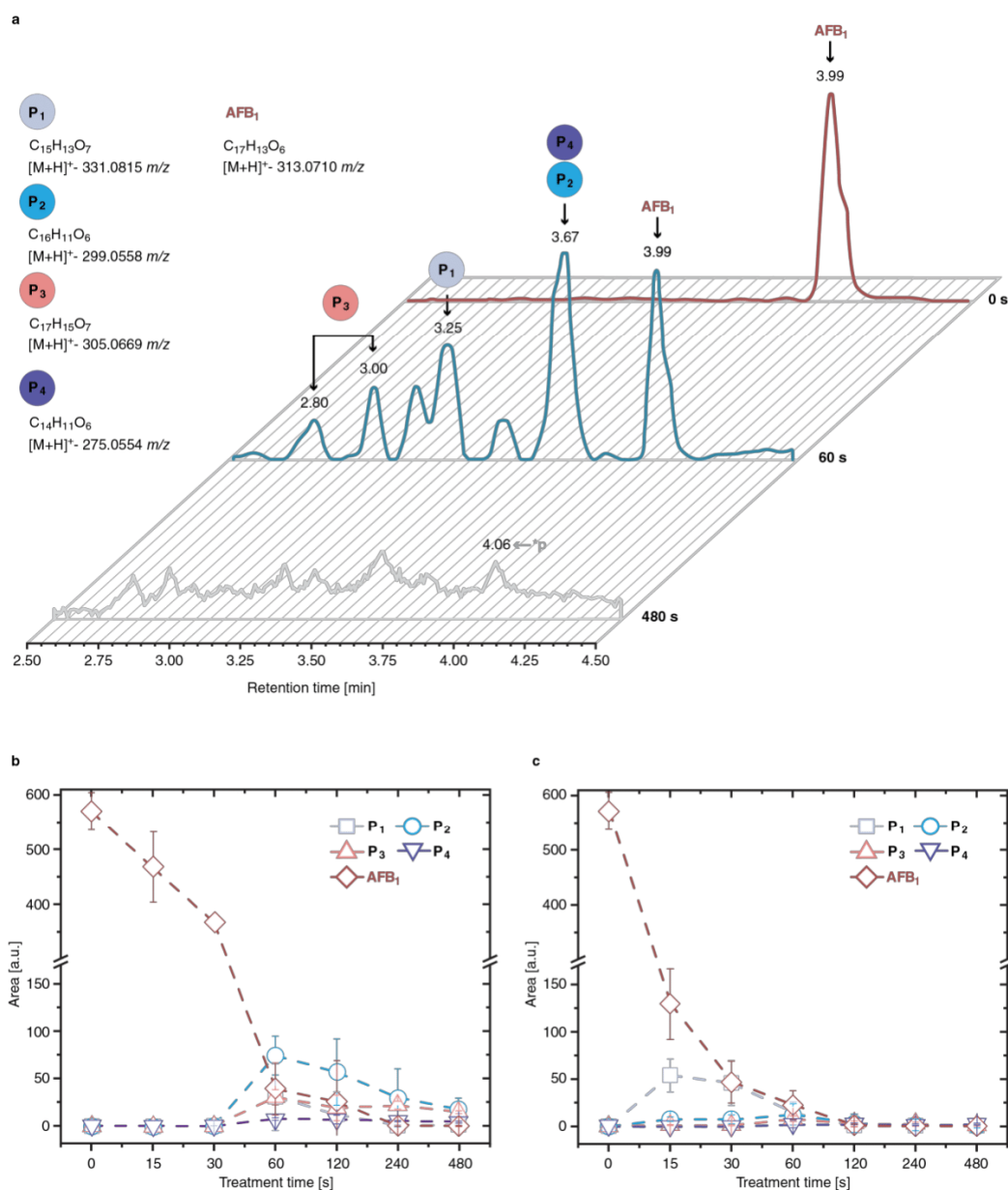
to a low dose of CAP RONS. In comparison to P₁, P₂ appeared to be more resistant, especially under low dose treatment conditions. According to its elemental composition, P₂ was reduced by one C atom and two protons in comparison to the parent AFB₁ molecule. The MS/MS fragmentation started with a single explicit removal of a carbonyl group, resulting in the first fragment ion at 271 *m/z*, which was followed by a second and third step of cleavages typical for AFB₁ (see Supplementary Fig. S5).

The largest peak area of product P₃ was observed after 60 s of treatment with a low dose of CAP RONS, where it remained present until 480 s. P₃ was found to have an elemental composition of C₁₅H₁₃O₇ corresponding to the molecular ion [M+H]⁺ - 305 *m/z*, which is one O atom more and two C atoms less than the chemical composition of the AFB₁ parent molecule. It was eluted at two different retention times (*i.e.* 2.80 and 3.00 min), which indicated that two different products with identical molecular mass may have been formed. However, this was shown not to be the case by MS/MS analysis, where an identical fragmentation pattern was observed regardless of the difference in the retention times, and was most likely a result of the LC-MS analysis conditions. MS/MS fragmentation started with the gradual cleavage of the fragment equivalent to formic acid. Starting with the intermediate removal of water, it resulted in the formation of a fragment ion at 259 *m/z*. Following this, the fragmentation pattern continued with gradual cleavages of carbonyl groups already observed in the MS/MS of AFB₁ (see Supplementary Fig. S6).

The fourth notable but minor degradation product was P₄, which strongly resembled the parental molecule of AFB₁. It began to develop in minor quantities after 60 s of RONS exposure regardless of the discharge conditions used and coeluted with P₂ at a retention time of 3.7 min. The exact mass of the protonated molecule corresponded to C₁₄H₁₀O₆ with [M+H]⁺ - 275 *m/z*. In comparison with the chemical composition of the AFB₁ molecular ion, P₄ was reduced by three carbons, one oxygen and two protons. The first fragment ion in the MS/MS spectrum was

observed at m/z 247, which is one carbonyl group less than the parental ion. Further, MS/MS fragmentation was comparable to the cleavages presented in the second and third step of the MS/MS fragmentation pattern of AFB₁ (see Supplementary Fig. S7).

LC-MS also revealed the existence of several other degradation products, in low quantities (p_x). One degradation product, p_1 , was found to comprise of a protonated molecular ion $[M+H]^+$ - 347 m/z with a chemical composition of C₁₇H₁₄O₈. With an elution peak at a retention time of 2.9 min, the highest concentration of p_1 was detected after 30 s of treatment following exposure to a high dose of CAP RONS. The first fragment ion, detected at m/z 329, was found to have one oxygen atom and two hydrogen atoms less than the AFB₁ parent molecule. After this first step, the fragmentation continued in multiple pathways. Nevertheless, the continuation of MS/MS fragmentation exposed the two most prominent ion fragments observed at m/z 301 and 273, matching the two serial cleavages of carbonyl groups (see Supplementary Fig. S8).



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189 **Figure 2 | UPLC characterisation of AFB₁ treated with CAP generated RONS.** **a** Chromatograms
 190 of untreated AFB₁, AFB₁ exposed to low dose CAP generated RONS for 60 s, indicating the formation
 191 of the four key degradation products, and AFB₁ exposed to low dose RONS for 480 s, where no AFB₁
 192 was detected. *p chromatogram peak with a retention time of 4.07 min does not correspond with AFB₁,
 193 rather a product with a molecular mass of 270 [M-Z] m/z and chemical composition $C_{15}H_{10}O_5$;
 194 chromatogram areas of AFB₁ and degradation products were obtained after the treatments with **b** low
 195 dose and **c** high dose CAP RONS.

Structural characterisation of degradation products by NMR. NMR spectra of AFB₁ samples exposed to CAP generated RONS for different periods confirmed degradation of the parent molecule in a time and discharge-power dependent manner. Intensities of characteristic NMR signals for protons H8 (δ_{H} 6.83 ppm, d, $J=7.0$ Hz) and H9 (δ_{H} 4.77 ppm, ddd, $J=7.0$, 2.3 and 2.3 Hz) next to the double bond of the terminal furan ring decreased exponentially with treatment time (see Fig. 3 for labelling scheme). These two signals fell below the limit of detection after 120 s of exposure to low-dose RONS conditions, and 60 s of exposure to high dose RONS. A significantly larger number of NMR signals were observed for samples treated with higher doses of RONS, indicating that the difference in RONS composition between the high and low dose CAP conditions can induce additional chemical reactions. In the ¹H NMR spectra of samples treated with a high dose of RONS, the appearance of broad signals in the region between δ_{H} 5.5 and 7.5 ppm indicated the possibility of the presence of larger molecules, resulting from radical chemistry induced polymerisation reactions. Despite these observations, NMR signals of several characteristic products were detected at both high- and low-dose RONS conditions and their concentrations were evaluated relative to the starting signal intensity of AFB₁ (see Supplementary Fig. S9).

The decrease of NMR signals of AFB₁ were accompanied by the emergence of new signals in the regions around signals of 2-OCH₃ and methylene groups at positions 2 and 3 of the parent molecule (see Supplementary Fig. S10). Correlations in 2D NMR spectra further confirmed that these signals belonged to similar structural elements and suggested that the degradation of AFB₁ proceeds mainly through the chemical modification of the two furanyl rings starting at the C8-C9 double bond, while the rest of the molecule is less affected. Using a combination of 2D NMR and mass spectroscopy, it was possible to characterise the four major degradation products emerging during CAP mediated AFB₁ decontamination (see Supplementary Table S1).

Starting at 30 s treatment times (*e.g.* samples exposed to 30 s of low dose RONS) signals appeared at δ_H 6.48 (d, $J=6.3$ Hz), 5.64 (d, $J=5.1$ Hz), 4.11 (dd, $J=9.5$ and 6.3 Hz), 2.36 (ddd, $J=13.4$, 9.5 and 5.1 Hz) and 2.22 (m) ppm, which are part of the same spin system according to TOCSY NMR spectra (see Supplementary Fig. S11). Observed chemical shifts and TOCSY correlations are consistent with the structure of 8-hydroxy-AFB₁, which corresponds to the MS/MS fingerprint of product P₁. Based on the intensity of signals in the ¹H NMR spectra, the concentration of the product remained low at all exposure times and fell below the limit of detection after 120 s. Similar observations were made for samples treated with high RONS concentrations, where ¹H NMR signals of 8-hydroxy-AFB₁ appeared after 15 s and were no longer observable for treatment times exceeding 30 s. This matches the degradation pattern of the parent molecule and indicates that 8-hydroxy-AFB₁ is an early transiently stable intermediate on the AFB₁ degradation pathway.

Following analogous analysis of NMR data, degradation product P₂ was identified as 9-methoxy-5,6-dioxo-5,6,7,8-tetrahydrocyclopenta[c]furo[2,3-h]chromene-3-carbaldehyde (Fig. 3). Compound P₂ exhibits two characteristic downfield shifted singlets in the ¹H NMR spectra at δ_H 10.32 (s) and 8.43 (s) ppm, which belong to the aromatic aldehyde proton H9 and proton H6 (Fig. 3). Correlations in ¹H-¹³C HMBC spectra connect these signals to C4 and C11 at δ_C 161 and 107 ppm, respectively. These are in turn correlated with the coumarin proton H3 at δ_H 7.21 ppm (s). A methoxy group signal at δ_H 4.02 ppm is also associated with the product and is correlated with the signal of C6 at δ_C 159 ppm in the ¹H-¹³C HMBC spectrum (see Supplementary Fig. S12). The signals of product P₂ appeared in ¹H NMR spectra of AFB₁ treated with low concentrations RONS for 30 s and persisted for all treatment times examined. They were also present in NMR spectra of samples treated with a high dose of CAP RONS from 15 to 120 s. Furthermore, product P₃ was assigned as 2-(7-hydroxy-9-methoxy-3,4-dioxo-1,2,3,4-tetrahydrocyclopenta[c]chromen-6-yl)acetic acid (Fig. 3). This compound is

characterised by a distinctive singlet at δ_H 3.67 ppm in the ^1H NMR spectrum, which belongs to a methylene group with a carbon signal δ_C at 29 ppm according to multiplicity edited ^1H - ^{13}C HSQC spectra. This proton signal is correlated to four carbons in the ^1H - ^{13}C HMBC spectrum with δ_C 173, 164, 157 and 104 ppm for 10-COOH, C4, C12 and C11, respectively, indicating that the methylene group is positioned between a carboxylic group and an aromatic ring. The downfield chemical shift of the aromatic carbon C4 is indicative of a C-O bond at that position. The methoxy group with a signal at δ_H 3.92 ppm was connected to the coumarin group of P_3 by a ^1H - ^{13}C HMBC correlation with the signal of C2 at δ_C 161 ppm (see Supplementary Fig. S12). Signal intensities in ^1H NMR spectra showed that P_3 is a high concentration product of AFB₁ degradation under the applied experimental conditions. The signal of the methylene group H10 at δ_H 3.67 ppm could be observed in spectra of samples treated for 30 s or more with low concentrations of RONS. Moreover, P_3 was already present in the treated samples after the shortest exposure time of a high RONS dose treatment, and it was fully degraded after exposure times longer than 240 s.

Aside from the signal of H9 in product P_2 , an additional weak signal characteristic of an aromatic aldehyde was observed at δ_H 10.32 ppm (s) in ^1H NMR spectra for most of the plasma treated samples. Its intensity correlates well with the intensity of the singlet at δ_H 6.52 ppm. These pair of signals could match a product in which the two furan rings of AFB₁ have been fully degraded, leaving a coumarin ring with a hydroxyl group at position 4 and an aldehyde group at position 11 (i.e. product P_4 , Fig. 3). The concentration of P_4 was low in most cases and slightly increased at long exposure times under high dose RONS conditions. This result matches the observations for the MS peak with the appropriate mass and seems reasonable for a heavily degraded product of AFB₁.

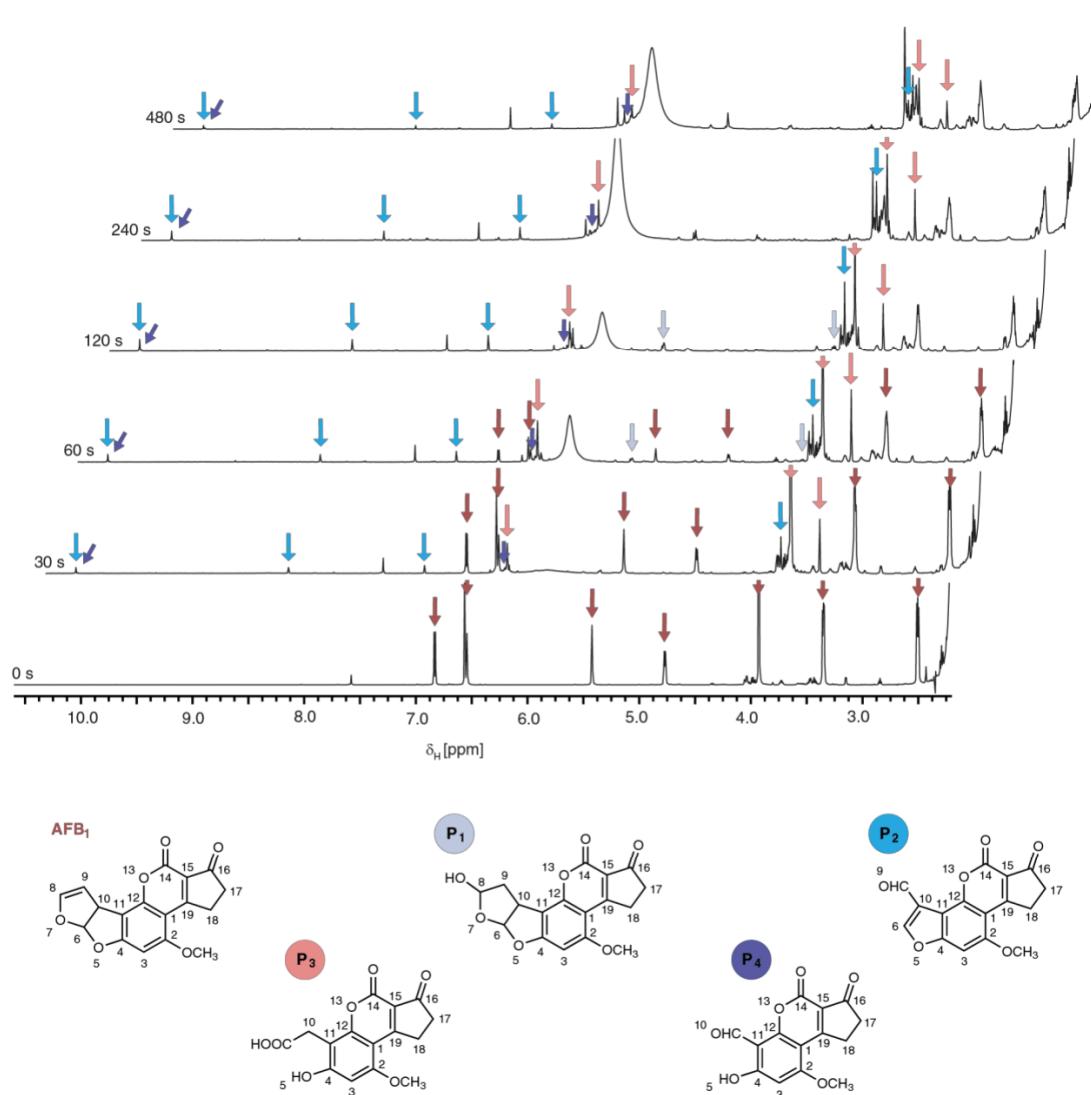
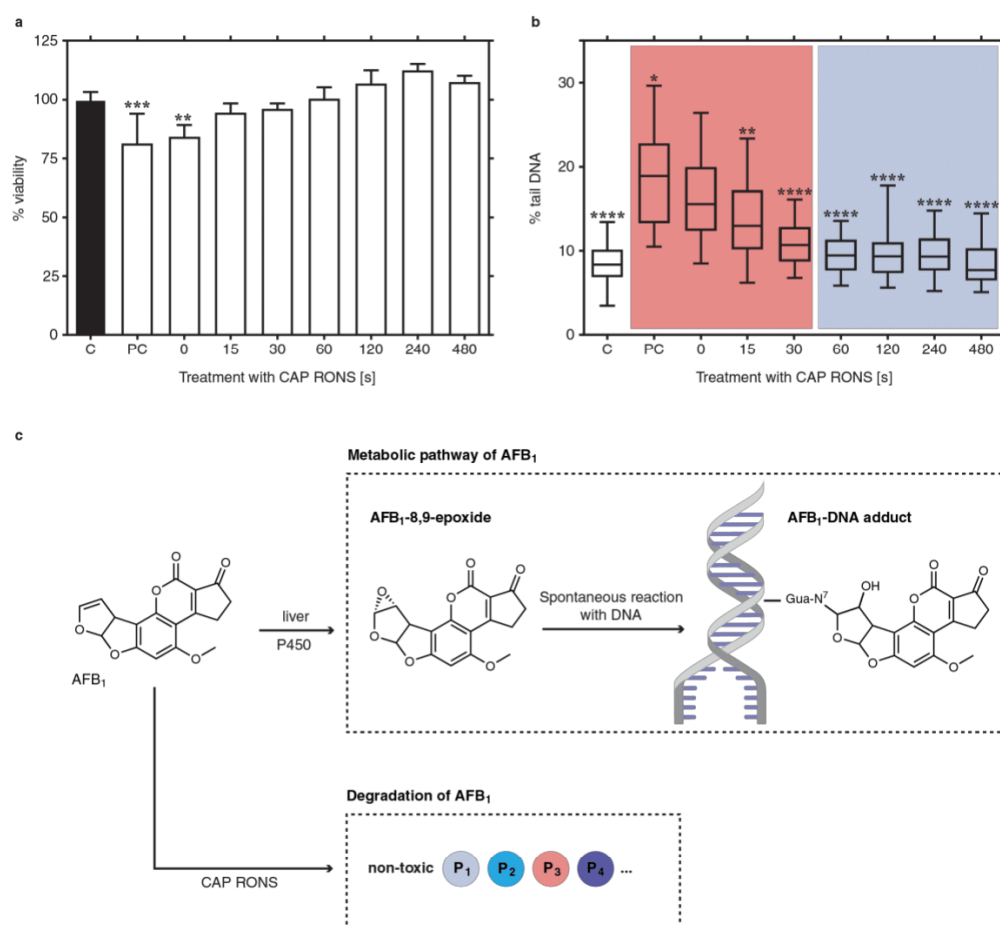


Figure 3 | ^1H NMR spectra of AFB₁ treated with low dose CAP RONS. Spectra were obtained in acetonitrile-*d* after different exposure time periods. Structures of AFB₁ and characteristic degradation products, which could be assigned from the NMR spectra are presented along with the atom numbering used in this paper. Characteristic ^1H NMR signals of each of the listed compounds are indicated by coloured arrows.

Evaluation of cytotoxicity and genotoxicity of CAP RONS treated AFB₁. Assessing the cytotoxic and genotoxic potential of any remaining AFB₁ degradation products is a vital step for the successful implementation and acceptance of the proposed approach. The cytotoxic effect of CAP treated AFB₁ on human hepatocellular carcinoma (HepG2) cells was determined using the MTS assay (Fig. 4a). The viability in the positive control with an AFB₁ concentration of 30 μ M was reduced to >70%. A similar result was observed in the case of non-treated AFB₁ (*i.e.* 0 s). In contrast, the reduction in viability was less notable or not present at all when HepG2 cells were exposed to RONS treated AFB₁. The most significant loss of cytotoxic potential was noted after exposure to a high dose of CAP generated RONS, where viability was comparable to non-treated cells (*i.e.* control).

The comet assay was used to evaluate the formation of DNA strand breaks in HepG2 cells as a result of the genotoxic activity of CAP exposed AFB₁ samples (Fig. 4b). Non-treated AFB₁ molecules generated a statistically significant increase in the formation of DNA strand breaks, which was comparable to the number of DNA strand breaks in the positive control. In contrast, CAP treated AFB₁ showed a decreasing trend in the formation of DNA strand breaks with increasing treatment time, regardless of the RONS dose used. In comparison to cells exposed to non-treated AFB₁, the reduction of strand breaks was most notable under high dose RONS conditions. Here a statistically significant decrease in strand breaks was observed after just 15 s of CAP exposure; following a further 45 s of exposure the formation of DNA strand breaks returned to values comparable to that of the control sample.



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Figure 4 | Reduction in AFB₁ toxicity following exposure to a CAP generated RONS. AFB₁ exposed to high concentrations of CAP generated RONS was applied to HepG2 cells for 24 hours. Controls were left unexposed. The positive control contained 30 μ M of AFB₁; **a** Cell viability was determined using an MTS assay. Three independent experiments, each containing five replicates, were performed. The results are presented as mean values $\pm$ SD. *P < 0.01; **P < 0.001 ***P < 0.0001 (one-way ANOVA, Dunnet post-tests); **b** The generation of DNA strand breaks after the exposure to treated AFB₁ was examined using the comet assay, and is presented as percentages of DNA in the comet tails. The distribution of the data is presented in box-plots. Three independent experiments were performed with fifty cells analysed in each experimental point. *P < 0.01; **P < 0.001 ***P < 0.0001 (two-way ANOVA, Dunnet post-tests); **c** schematic of the normal metabolic pathway of AFB₁ resulting in genotoxicity and the degradation of AFB₁ with CAP RONS leading to the loss of its toxicity.

Discussion

Plasma as an efficient tool against AFB₁. This study, based on a comprehensive experimental investigation of CAP mediated AFB₁ degradation, demonstrates the outstanding potential of technique to combat mycotoxin contamination. An AFB₁ decontamination efficiency of 96% was achieved after only 60 s of treatment, with no residuals detected after 120 s of exposure. Notably, the initial concentration of AFB₁ used in these investigation was 35 µg/ml (*i.e.* 35 µg/kg in conversion), which is 8.75 times higher than the maximum permitted concentration allowed in cereals and foods derived from cereal origin (*i.e.* 4 µg/kg) within EU legislation.¹⁷ In consideration of this, a 60 s CAP treatment was sufficient to decrease the AFB₁ concentration to acceptable levels (*i.e.* to 1.5 µg/kg). The CAP method employed in this study is therefore significantly more efficient than other reported mycotoxin decontamination approaches including the preliminary investigation conducted using plasmas.^{15,18,19}

The ability of CAP to degrade chemical compounds is generally attributed to the synergistic production of RONS, the generation of UV photons and thermal effects. In this investigation, a thermocouple probe confirmed that within the first 60 s of treatment, where the primary degradation occurred, the surface temperatures never exceed 40 °C. Hence thermal effects can be neglected as AFB₁ molecules are thermally stable.¹⁶ Furthermore, a direct comparison of the CAP treatment with UV-C irradiation demonstrated the significantly better decontamination performance of CAP, achieving a notable level of AFB₁ degradation after only 30 s of treatment (Fig. 1). Conversely, the equivalent duration of exposure to UV-C irradiation did not change the initial AFB₁ concentration. Furthermore, the photons produced by the UV-C lamp are primarily emitted from mercury at 254 nm (see Supplementary Fig.S1a). Thus, they are more energetic than the UV photons from the plasma (see Supplementary Fig.S1a, plasma emission primarily from nitrogen molecules, 300 – 400 nm). Given the limited

impact of the UV-C treatment it is unlikely that the UV from the plasma played a direct role in the degradation process. Based on these findings, CAP generated RONS must be the crucial agents responsible for mycotoxin degradation. Due to the nature of the CAP device used in this investigation, short-lived RONS such as atomic oxygen (O) and hydroxyl radicals (OH) are known to react beyond the visible discharge region to form less-reactive species, *e.g.* ozone (O₃), hydrogen peroxide (H₂O₂), nitric oxide (NO) and other nitrogen oxides (NO_x), and nitrous (HNO₂) and nitric acid (HNO₃).^{20,21} Taking into consideration the 5 mm separation between the treated sample and plasma region, it is clear that long-lived RONS are the major decontamination agents responsible for AFB₁ degradation. Moreover, the composition of RONS generated by CAP can be electrically manipulated since they depend on the power dissipated within the discharge. This is observed from the FTIR absorption analysis performed during CAP treatments (see Supplementary Fig. S1b). In the simplified case, a low discharge power generated a low dose of CAP RONS, consisting primarily of reactive oxygen species (ROS), with O₃ observed to dominate the absorption spectra. In contrast, high discharge power conditions led to the generation of a high dose of RONS, which showed a rapid transition from an ROS-dominated to an RNS-dominated mode of operation, consisting primarily of the oxides of nitrogen (*e.g.* N_xO_y). The ROS-RNS transition has been extensively studied both experimentally and computationally and is attributed to the accelerated production of NO due to the presence of vibrationally excited nitrogen at elevated discharge powers and the subsequent poisoning of O₃.²² Notably, the use of O₃ for AFB₁ degradation has been widely investigated and found to be considerably less efficient than the CAP system reported here.⁹ Given that the treatments were performed with moist AFB₁ samples containing water and acetonitrile, the long-lived gas phase RONS produced by CAP cannot be considered in isolation, their subsequent reactions at the gas-sample interface must be considered. Interaction of CAP generated RONS with a protic solvent such as water namely leads to unique liquid

physicochemical modifications. Similar to the gas phase chemistry, the composition of aqueous phase RONS strongly depends on the type of discharge, its physical properties and the chemical composition of the surrounding environment, including the type of gas and liquid used. CAP generated RONS react at the gas-liquid interface to form aqueous phase species such as H_2O_2 , NO , NO_2 , HNO_2 and HNO_3 . Ions such as nitrites (NO_2^-) and (NO_3^-) are further generated through hydrolysis of HNO_2 and HNO_3 , and through NO and NO_3 interaction with water. Correspondingly, the number of protons (H^+) is increased, resulting in a reduction of pH, which limits the diffusion of O_3 into the liquid. Within the aqueous phase, further interactions between RONS are able to induce the generation of reactive species such as OH , hydroperoxyl radical (HO_2) and peroxyxynitrous acid (ONOOH), which are known to be powerful oxidants.^{23,24}

RONS induced mechanisms of AFB₁ degradation. The generated RONS possess a high oxidative potential with high affinity to react with vinyl bonds in organic compounds. From the results, it was observed that the degradation of AFB₁ starts at the targeted double bond between C8 and C9. This was confirmed with NMR analysis, which additionally indicated that the coumarin part with cyclopentonyl and methoxy group of AFB₁ molecule remained preserved in all important degradation products. The reaction pathways showing the various intermediaries are summarised in Fig. 5.

The generation of P_1 implied that RONS modification of AFB₁ began with electrophilic addition of water on C8. This resulted in the formation of its 8-hydroxy derivate or hemiacetal, also known as AFB_{2a}. This is a well recognised product of AFB₁, which is produced enzymatically during biotransformation as well as non-enzymatically under acidic conditions.²⁵

Despite this, CAP mediated modification of the AFB₁ molecule is also initiated through sequent radical reactions. Given the potential for the generation of short-lived RONS

within aqueous media, it is assumed that the wet AFB₁ samples were subjected to highly reactive short-lived species such as OH. The initial step of OH interaction included the H-atom abstraction from the bonds C-H at the 8- and 9- positions of AFB₁, expectedly resulting in the formation of its unstable 8,9-dialdehyde intermediate.²⁶ No similar product was detected with LC-MS or NMR, suggesting that the 8,9-dialdehyde rapidly decayed through protonation of the oxygen at 8-position, which was catalysed by CAP-induced acidic conditions (HNO₂, HNO₃) and increased concentrations of protons. Following this, the formic acid was eliminated, forcing the electrons in the remaining molecule to rearrange and form a double bond between C6 and C10. This resulted in the new stable aromatic aldehyde P₂. Additionally, P₂ was also generated by radical reactions involving P₁; H-atom abstraction from 9-position resulted in the unstable 8-carbonyl-9-hydroxy intermediate, which was followed by additional oxidation of 9-position and already acknowledged elimination of formic acid. In another path, O₃ could also be potentially involved in the formation of the P₂ since it is known to react with vinyl bonds through Criegee oxidation. However, the outcome of such a reaction in the oxidative workup would most probably include the formation of carboxylic functional groups at 8- and 9-positions. No such product was observed. Thus, O₃ was not directly involved in the initial steps of CAP RONS degradation of AFB₁.

The presence of RONS such as H₂O₂ and HO₂ influenced another degradation pathway of AFB₁. Peroxide reaction with vinyl bond proceeds in the generation of epoxides. Thus, it was assumed that unstable AFB₁-8,9-*exo* epoxide was formed after the exposure to CAP RONS. Further spontaneous hydrolysis of the epoxide in the CAP RONS induced acidic conditions resulted in the generation of *trans* AFB₁ -8,9-diol, which corresponded to p₁. Moreover, diol was formed as one of the products of OH reaction with P₁ as well. Unfortunately, the small quantities of p₁ prevented the identification of the molecule with NMR analysis. Nevertheless, the MS/MS fragmentation of p₁ coincided with the AFB₁-8,9-diol

identified in the study by Johnson *et al.*²⁷ The p_1 is a hemiacetal, which can be transformed to a complementary difuranyl-ring-opened oxyanionic AFB₁ α -hydroxydialdehyde form. The conversion into a hydroxydialdehyde is more significant under slightly alkaline conditions.²⁸ This conversion was present in a smaller percentage in the conditions containing CAP RONS. Nevertheless, the hydroxydialdehyde molecule could potentially correspond to the unidentified aldehyde signals in NMR.

In the open hydroxydialdehyde form, CAP RONS could have also reached inner positions in the difuranyl part with P₃ and P₄ as a possible outcome. The formation of the P₃ initiated with the protonation of the 5-position, succeeding with the RONS induced cleavages of both aldehyde parts at 8- and 6-positions. Simultaneously, C9 was further oxidised into a carboxyl group. The generation of carboxylic acids in radical chemistry could be namely induced by different RONS, including OH and O₃.^{26,29}

Lastly, P₄ was generated as a terminal degradation product after extended exposure of AFB₁ to CAP generated RONS. This product was formed either by further radical degradation of P₃ or by an alternative degradation pathway initiating from an open hydroxydialdehyde form of p_1 . In the first scenario, the attack of radicals such as OH induce the conversion of the acid into its anion, which is then subjected to the electrolysis, generating unstable carboxylic radicals. Next, the cleavage of carbon dioxide occurred, leading to the radical formation at 10-position. The intermediate product abstracted oxygen, which resulted in the formation of stable aldehyde product known as P₄. Alternatively, the generation of P₄ started from p_1 , where CAP RONS induced the cleavages of C6, C8 and C9 position, giving the equal C10 radical, which was then oxidised to the aldehyde.

Reducing the toxicity of AFB₁. The principal toxic activity of AFB₁ refers to its ability to bind with DNA and induce a mutation in the p53 tumour suppression gene.³⁰ When ingested into a

living organism, AFB₁ is transported into the liver, where it is metabolised by phase I enzymes such as cytochrome P450 1A2 and 3A4. Once bioactivated into its most potent metabolite, AFB₁-8,9-exo epoxide, it intercalates with 5' ends of guanines in the DNA strand, which disrupts the covalent bonding with the guanyl N7 atom. This process leads to the generation of stereospecific positively charged *trans*-8,9-dihydro-8-(N⁷-guanyl)-9-hydroxyafatoxin B₁ (AFB₁-N⁷-Gua) as the primary AFB₁-DNA adduct and its hydrolysed imidazole ring-opened AFB₁ formamidopyrimidine (FAPY) adduct. FAPY is notably more resistant to DNA repairing mechanisms and therefore predominantly responsible for latter AFB₁ related mutagenic events.³¹ Apart from this, AFB₁-8,9-exo epoxide is also spontaneously hydrolysed to *trans* AFB₁-8,9-diol (p₁), which is reversibly reorganized into AFB₁ hydroxydialdehyde, especially in slightly basic conditions typical of the liver cell environment.²⁸ Simultaneously, AFB₁ is metabolized into its hemiacetal form AFB_{2a} (P₁). As AFB₁ diol, higher pH values promote its conversion to the open AFB_{2a} dialdehyde form.²⁵ The removal of a critical double bond in AFB₁ terminal furan ring contributed to the reduced mutagenicity of both products, however, their opened dialdehyde ions are believed to form Schiff bases with primary amine groups in proteins, resulting in protein adducts responsible for the additional cytotoxic effect of AFB₁.^{25,28}

From the perspective of chemical analysis, we demonstrated that CAP generated RONS eliminate the critical vinyl bond between C8 and C9 in the molecule of AFB₁. CAP modification of the AFB₁ active site induced the formation of both cytotoxic AFB_{2a} (P₁) and AFB₁-diol (p₁), which were subsequently degraded, in a similar manner to AFB₁. According to these findings, it can be hypothesised that CAP is able to successfully detoxify AFB₁ molecules. To confirm this, CAP treated AFB₁ samples were investigated with toxicological tests using a HepG2 cell line that represents a major target of AFB₁ toxicity and expresses metabolic enzymes required for AFB₁ transformation to the reactive metabolites.³² The MTS

assay revealed that the cytotoxicity of the treated AFB₁ was reduced and reached the levels comparable to those of the control samples in a matter of seconds. In comparison to cytotoxicity testing performed in other studies focused on alternative techniques, the CAP treatment was significantly more efficient. For example, the viability of human embryo hepatocytes (L-02 liver cells) was normalised only after 30 min of exposure to UV irradiation.³³ Similarly, a maximal 87 % viability of HepG2 cells was achieved when following electron beam irradiated of aqueous AFB₁ samples.³⁴

In addition to the determination of cytotoxicity of CAP RONS treated AFB₁, the reduction in genotoxic potential was examined. In contrast to the MTS assay, 60 s of AFB₁ treatment with CAP was required to reduce DNA single-strand breaks to a level comparable with the control sample. This approach has not been applied to AFB₁ treated by alternative methods; however, an insignificant reduction of mutagenicity was reported for *Salmonella typhimurium* following electron beam irradiation of AFB₁.³⁴ Compared to the results in this study, CAP RONS are undoubtedly far more effective.

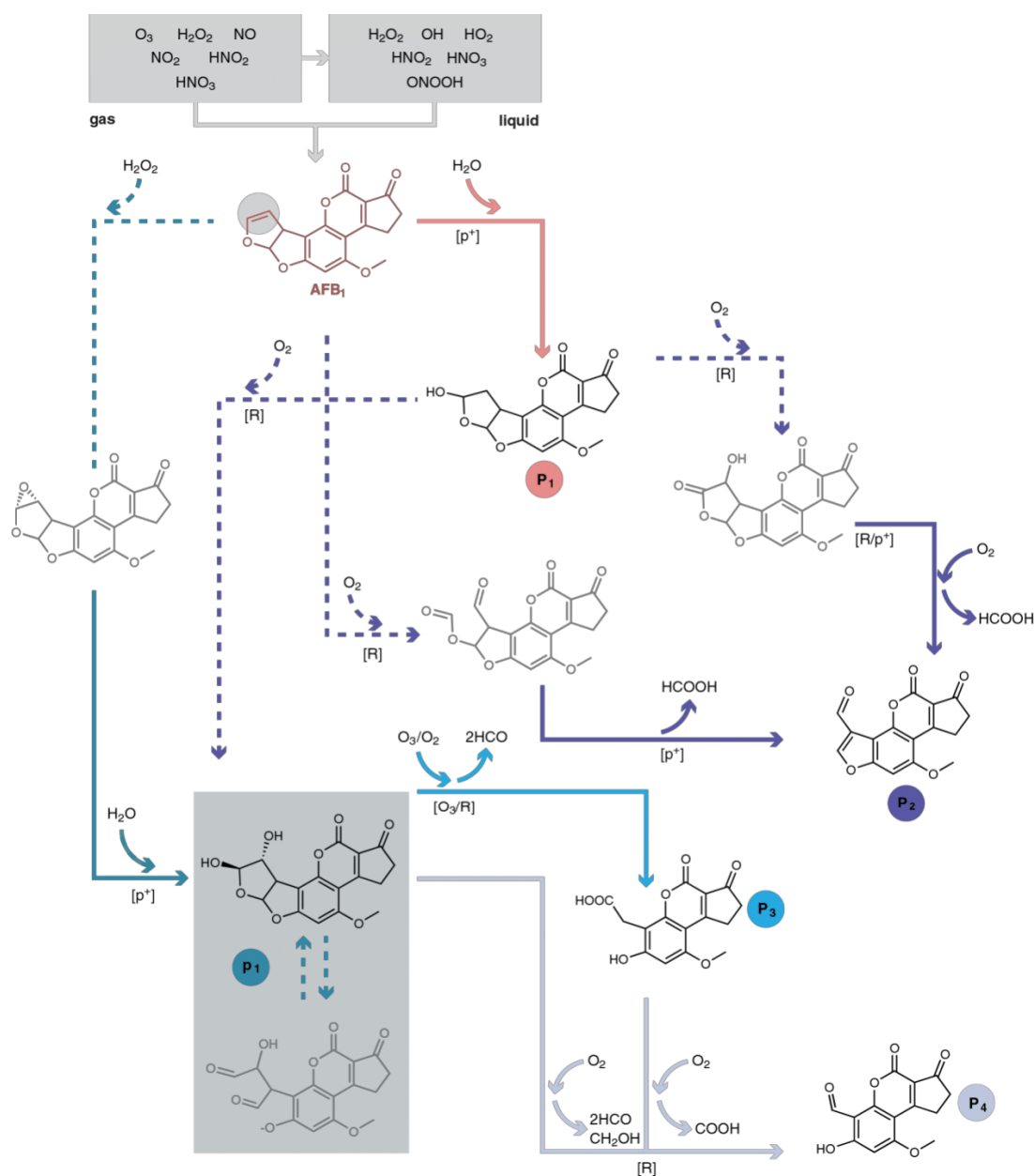


Figure 5 | Proposed CAP RONS mediated degradation pathways of AFB₁. Hydration of AFB₁ and creation of 8-hydroxy derivate (P₁). Further radical attack on AFB₁ and P₁ resulting in possible intermediate products such as 8,9-dialdehyde derivate and 8-carbonyl-9-hydroxy derivate. Further elimination of formic acid and generation of a stable aldehyde (P₂) with a new aromatic system, which includes an inner furan ring of AFB₁. Peroxide induced AFB₁-8,9-*exo* epoxide formation and its transformation to AFB₁-8,9-diol in acidic conditions. The diol or hemiacetal form can be converted to its open hydroxydialdehyde ion, which enables RONS to attack the inner atoms of the difuranyl part of

AFB₁. Further oxidation of 10-position to the carboxyl group, forming P₃. RONS induced elimination of formic acid and oxidation of 6-position to another aromatic aldehyde (P₄) as a terminal product of CAP RONS degradation of AFB₁.

Conclusions

In this study, we provide a novel insight in to the degradation pathways of AFB₁ initiated by exposure to CAP generated RONS. AFB₁ is a potent toxin, mutagen, and carcinogen and poses which can be found on contaminated peanuts, cottonseed, corn, and rice. Despite this, no current decontamination approach has proven capable of efficiently removing it from the food supply chain. Here we demonstrate that not only is CAP significantly more effective than competing approaches, but the RONS mediated degradation pathways ultimately lead to a complete loss of toxicity. We highlight that AFB₁ degradation is always initiated by rapid scission of the vinyl bond between 8- and 9-position on the terminal furan ring of AFB₁, proceeding via multiple degradation pathways to the ultimate degradation of the molecule. The technology carries a tremendous potential to transform the future agriculture and food industry, where classical approaches with wet chemistry, thermal processing or UV light irradiation have been unsuccessful. While the focus of this study has been on identifying the breakdown products and pathways leading to a reduction AFB₁ toxicity when exposed to CAP RONS, demonstrating the real-world potential of CAP treatment is also a vital step. The CAP system used in this study has been extended to other types of mycotoxins and real cases including the treatment of contaminated corn kernels (see Supplementary Fig. S17, S18). The degradation results for other mycotoxins on realistic substrates follow a similar trend to the case of AFB₁ and thus further highlight the utility of our approach. Ultimately, this work provides the crucial insight necessary to accelerate the development of an extremely promising non-thermal mycotoxin decontamination technology.

Materials and Methods

Preparation of AFB₁ and CAP treatments. AFB₁ was obtained from ACROS ORGANICS (Thermo Fisher Scientific; New Jersey, USA). The AFB₁ stock solution (S) with concentration 0.5 mg/ml was prepared in acetonitrile. Prior to CAP treatments, S was diluted with deionised water (diH₂O) in proportion S:diH₂O 2:1. 100 µL of diluted S containing 33 µg AFB₁ was deposited on glass coverslips and left 5 min to dry. Following this, AFB₁ was exposed to CAP generated RONS for periods 15, 30, 60, 120, 240 and 480 s. The distance between the plasma generation point and the sample was kept constant at 5 mm. To generate different concentrations and compositions of RONS, CAP was generated with low and high discharge powers of X W.cm⁻¹ and Y W.cm⁻¹, respectively. Discharge power is approximately proportional to the concentration of RONS generated by the plasma. In addition, after 480 s of plasma generation, the gas phase effluent of the low power discharge contained mostly ROS, whereas RNS were found to be prevalent under high power plasma conditions. Further details on the ROS to RNS mode transition can be found in the supplementary material (see Supplementary Fig. S1b). Following CAP exposure, samples were diluted in various solvents required for further analysis.

Determination of degradation products and degradation pathway.

LC-MS and LC-MS/MS analysis of degradation efficiency and degradation products. LC-MS measurements were performed with a Water Acquity binary pump based ultra-performance liquid chromatography (UPLC) system (Waters, Milford, USA) coupled with a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF) equipped with an orthogonal Z-spray electrospray (ESI) interface (Waters Micromass, Manchester, UK). CAP RONS treated AFB₁ samples were diluted in acetonitrile and run through a C18 Ascentis Express (50 x 2.1 mm; 2 µm particles, Supelco) LC column with mobile phases consisting of 0.1 % formic acid in water (A) and acetonitrile (B). The elution gradient was produced by a linear increase from 10% B to 60% B in 6 min and then back to initial conditions. The total run time of the conditioning of the column and the sample analysis was 7 min. Compressed nitrogen was used as both nebulising and drying gas, with nebuliser gas flow rate around 20 L h⁻¹ and de-solvation gas flow rate up to 600 L h⁻¹. A cone voltage of 30 V and a capillary voltage of 3.0 kV were used in positive ion mode. The de-solvation temperature and source temperature were set to 300 °C and 130 °C. To obtain accurate mass measurements and the determination of the elemental composition of the target ions, a mass resolution was set to approximately 9000. The mass spectra was collected in centroid mode over a range of 50 – 1000 m/z with a scan time of 1 s and interscan time of 0.1 s. Furthermore, comparable conditions were used in tandem mass spectrometric (MS/MS) analysis of selected ions at 20 V collision energy. Mass Lynx v4.1 (Micromass, Manchester) was used as data station operating software.

NMR. Samples for NMR spectroscopy were dissolved in acetonitrile-d₃ and transferred to 5 mm NMR tubes. ¹H NMR spectra were recorded to follow the formation of degradation products following exposure of samples to CAP. Additionally, 2D ¹H-¹³C HSQC, ¹H-¹³C HMBC and ¹H-¹H TOCSY spectra were recorded for samples containing higher concentrations of characteristic degradation products to enable their characterisation. All spectra were recorded at 25 °C on an Agilent Technologies DD2 600 MHz NMR spectrometer and Agilent-Varian VNMRs 800 MHz NMR spectrometer equipped with Cold Probes. Chemical shifts were referenced to the solvent residual signal (δ_H 1.93 ppm, δ_C 1.3 ppm) and are reported relative to TMS.

Cytotoxicity and genotoxicity in human hepatocellular carcinoma HepG2 cells.

Cultivation and exposure of HepG2 cells. HepG2 cells were grown in Minimum Essential Medium Eagle medium (Sigma-Andrich, St. Louis, Missouri, USA) supplemented with 10 % FBS, 1.5 mM L-glutamine (Sigma-Andrich, St. Louis, Missouri, USA), 1% non-essential amino acids, 16 mM sodium bicarbonate and 1% penicillin/streptomycin at 37 °C in 5% CO₂. The cells were used between passages six and nine. In short, before the exposure to plasma treated AFB₁ samples, cells were cultured for 24 h to attach. Plasma treated AFB₁ samples and non-treated AFB₁ (0 s) were diluted in the previously described culture media containing 1% DMSO (Sigma-Andrich, St. Louis, Missouri, USA). AFB₁ in a concentration of 30 µM was used as a positive control; the concentration of non-treated AFB₁ samples was approximately equivalent to the concentration of the positive control. The control samples with non-treated cells contained culture medium with 1% DMSO.

Cytotoxicity assay (MTS assay). The cytotoxic effect of CAP treated AFB₁ samples on HepG2 cells was determined with a MTS tetrazolium reduction assay (Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, USA), according to the manufacturer's instructions, with minor modifications. Briefly, cells were plated on 96-well plates with 8000 cells per well and incubated for 24 h to attach. Following this, the growth medium was replaced with growth medium containing CAP treated AFB₁ and incubated for a further 24 h. After the exposure, 40 µL of freshly prepared MTS:PMS solution (20:1) was added to each well and incubated for 3 hours in the dark to form the coloured MTS formazon product. Finally, the absorbance was measured at a wavelength of 490 nm using a Microplate reader (Synergy MX, BioTek, Winooski, VT, USA). Cell viability was calculated by comparing the absorbance values of the wells containing the cells exposed to CAP treated AFB₁ with those of unexposed cells and presented as % of cell viability. Three independent experiments were performed, each time with five replicates per treatment point.

Single cell electrophoresis assay (comet assay). HepG2 cells were plated into 12-well cell culture plates (Corning Costar Corporation, Corning, USA) with a density of 80000 cells per well and incubated for 24 h to attach. The

growth medium was then replaced with fresh growth medium containing CAP treated AFB₁ and incubated for a further 24 h. After the exposure, the cells were harvested for the comet test, which was performed following the protocol of Singh *et al.*³⁵ with minor modifications.³⁶ Firstly, 30 µL of cell suspension was mixed with 70 µL 1% LMP agarose and put on fully frosted slides pre-covered with 80 µL of 1% NMP agarose. Afterwards, the cells were lysed (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 hour at 4 °C, which was followed by DNA denaturation (300 mM NaOH, 1 mM EDTANA₂, pH 13) for 20 min at 4 °C and electrophoresis for 20 min at 25 V and 300 mA. Lastly, the slides were neutralised in 0.4 M Tris buffer (pH 7.5) for 15 min, stained with GelRed Nucleic Acid Stain (1:3333) and analysed with a fluorescence microscope (Nikon, Eclipse 800). Images of 50 randomly selected nuclei per sample were analysed with the software Comet Assay IV (Perceptive Instruments, UK) and the DNA damage was expressed as % of DNA in the comet tail. For each CAP treated AFB₁ sample, three independent experiments were carried out. Two-way ANOVA was used to analyse the statistically significant differences between samples within each experiment, using a Dunnet's test for multiple comparisons of treated and non-treated samples.

Data availability

Full data supporting the findings of this study are available within the article and Supplementary Information, as well as from the corresponding authors upon reasonable request.

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Reactive oxygen and nitrogen species for targeted reduction of mycotoxin toxicity

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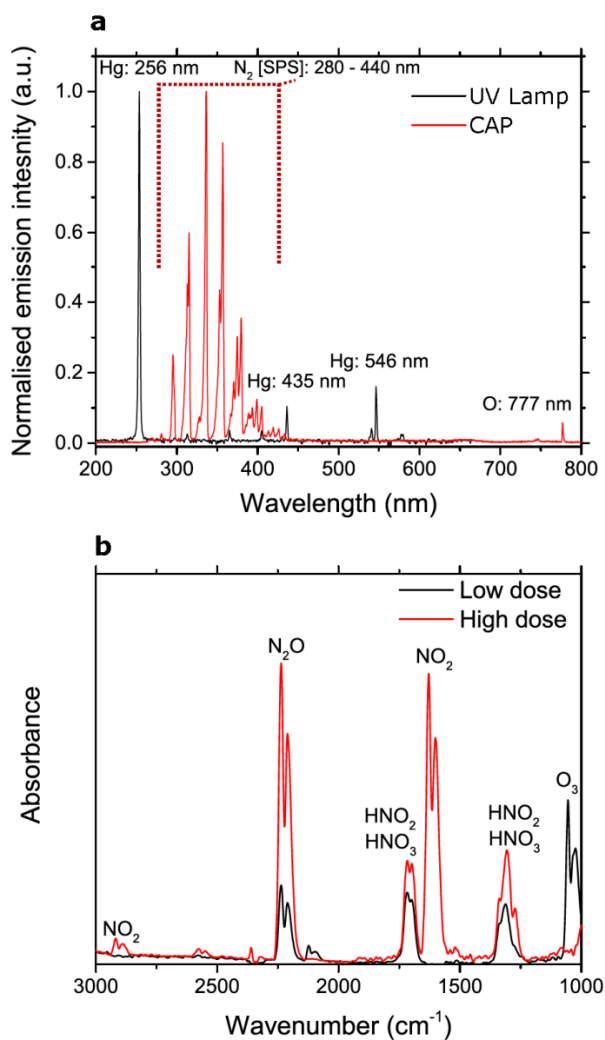
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718 I Plasma diagnostics



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720 **Supplementary figure 1 | Optical diagnostics of plasma and UV systems. a** Optical

721 emission spectroscopy of UV lamp and CAP system operating under high dose RONS

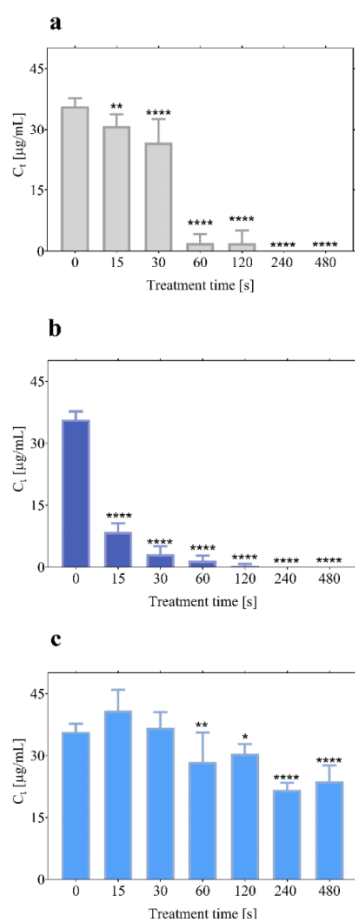
722 conditions; **b** FTIR absorption spectra of CAP system operating under low and high RONS

723 dose conditions.

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727 **II Decontamination efficiency**

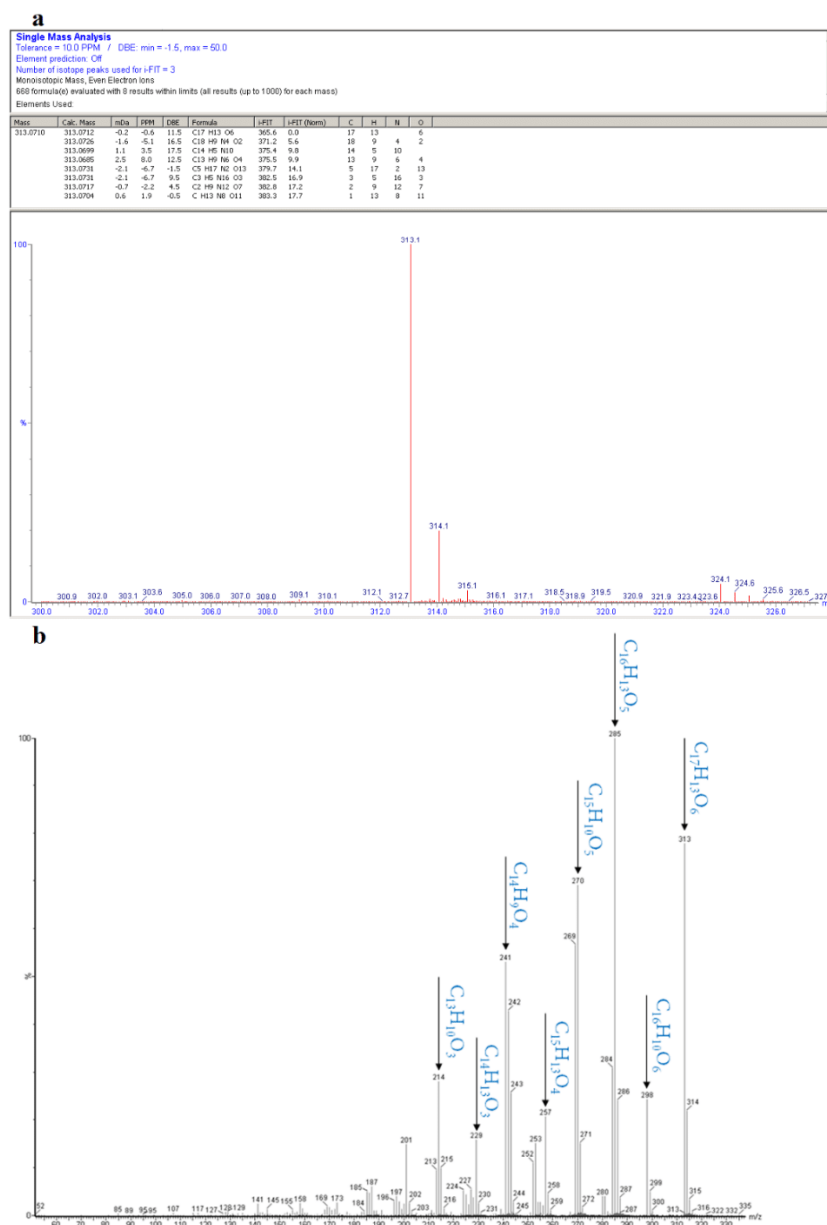
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729 **Supplementary figure 2 | AFB₁ decontamination efficacy of CAP RONS and UV-C**730 **irradiation.** Concentrations of AFB₁ residuals obtained after the exposure to **a** low dose CAP731 RONS, **b** high dose CAP RONS, and **c** UV-C irradiation. The concentrations of AFB₁ were

732 determined with LC-MS. A calibration curve was calculated from the areas of the dilutions of

733 AFB₁ standard with known concentrations. The tests were performed in multiple independent734 experiments and replicates. The results are presented as mean values $\pm$ SD. *P < 0.01; **P <

735 0.001 ***P < 0.0001 (one-way ANOVA, Uncorrected Fisher's LSD post-tests; n>3).

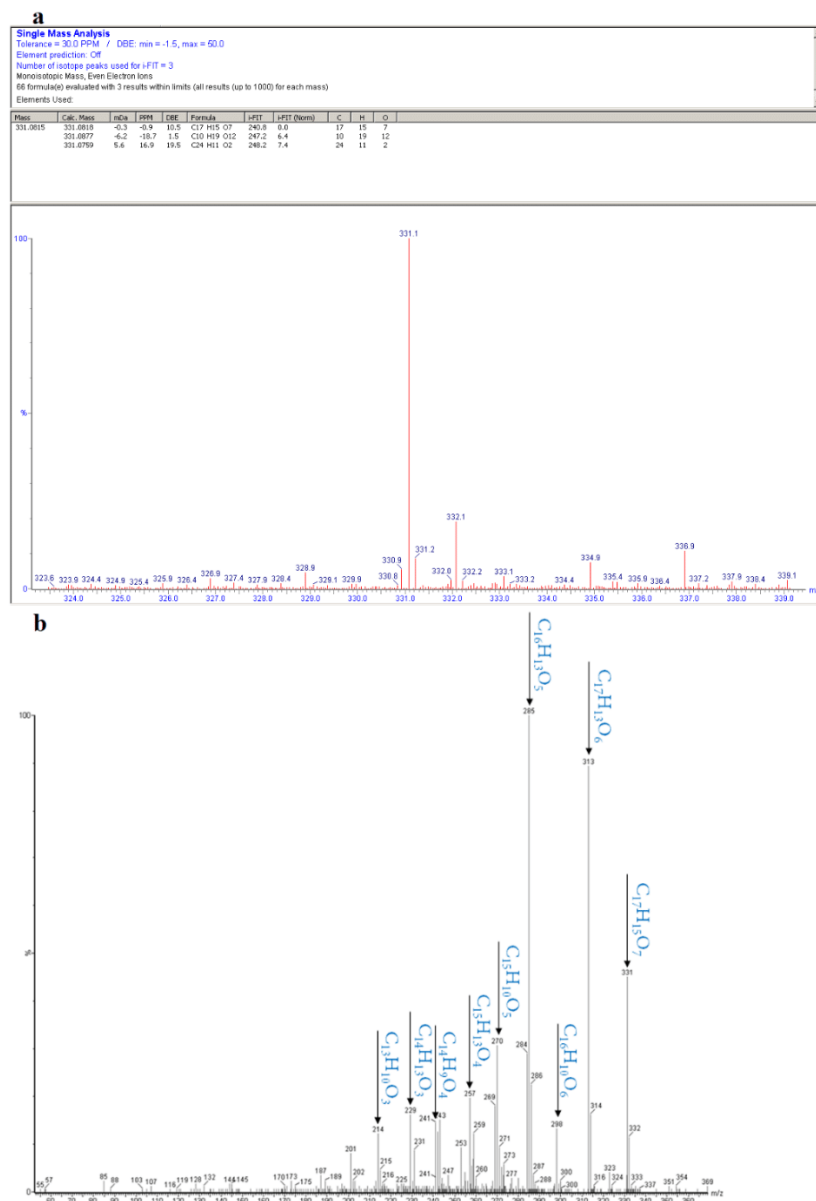
736 **III HRMS**

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738 **Supplementary figure 3 | HRMS of AFB₁.** **a** Result of HRMS measurements of $[M+H]^+$ at

739 m/z 313; **b** MS/MS spectrum of parent ion: $[M+H]^+$ at m/z 313. Prevalent fragment ions are

740 observed at m/z 298, 285, 270, 257, 241, 229 and 214.

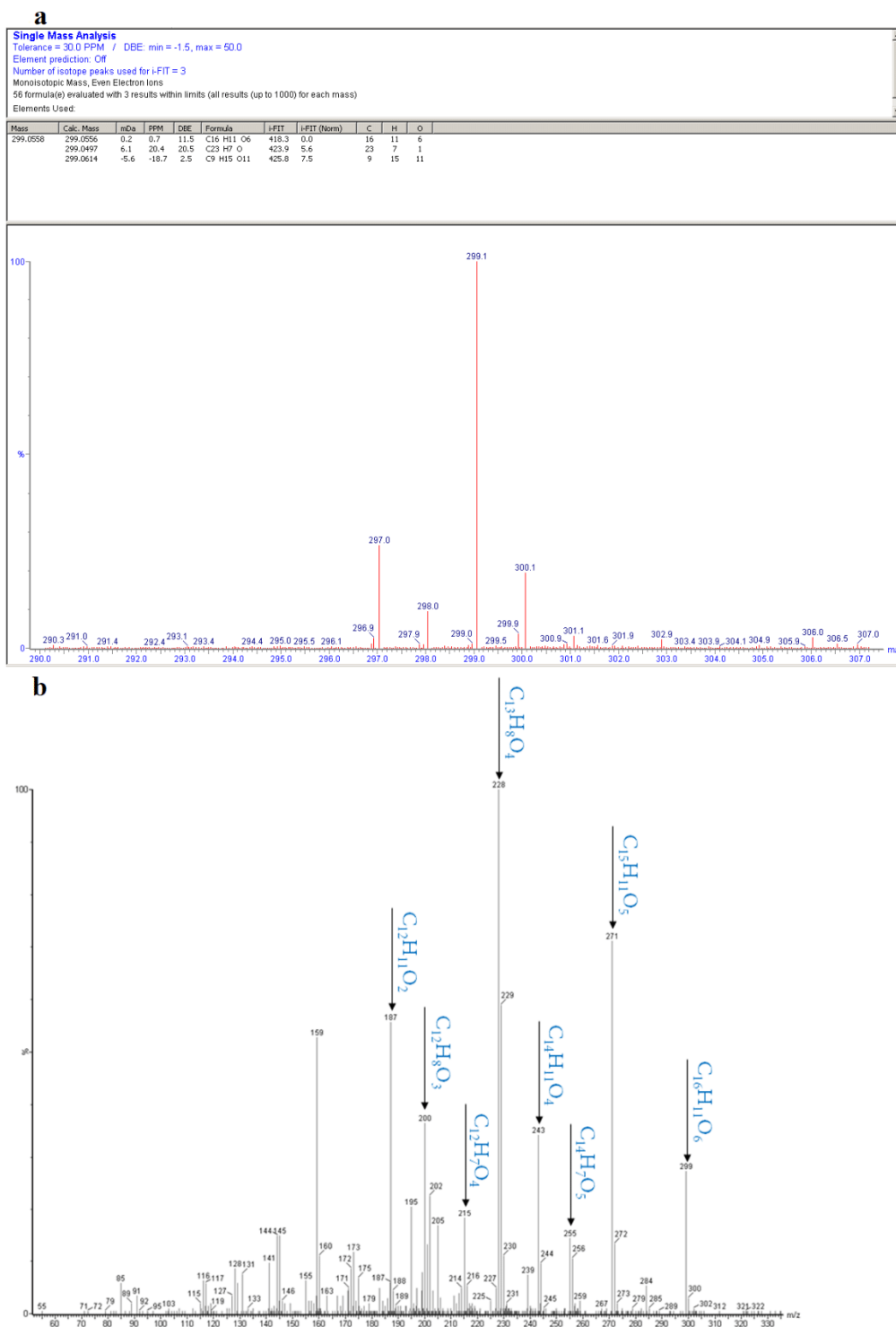


741

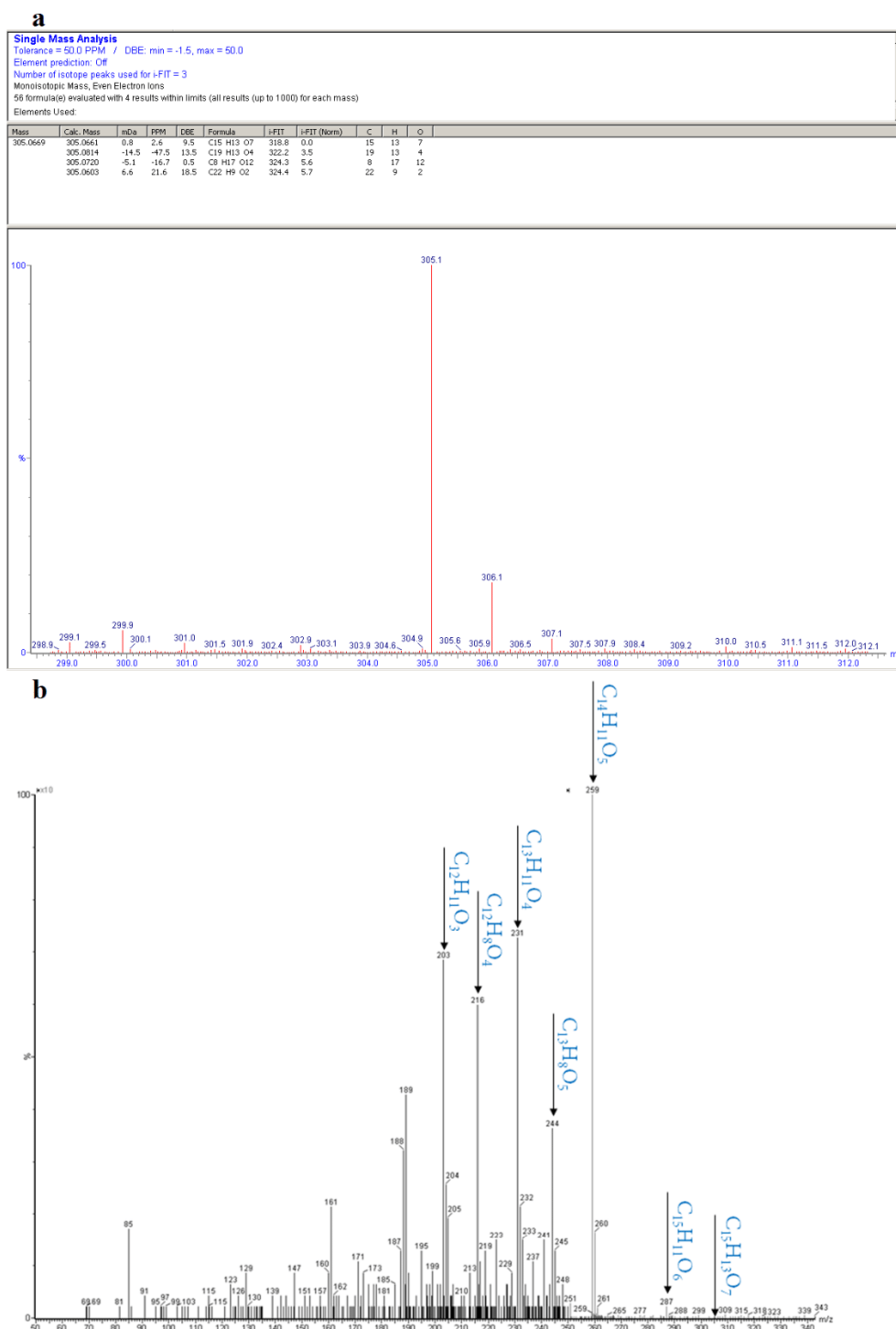
742 **Supplementary figure 4 | HRMS of P1. a** Result of HRMS measurements of $[M+H]^+$ at m/z

743 331; **b** MS/MS spectrum of parent ion: $[M+H]^+$ at m/z 331. Prevalent fragment ions are

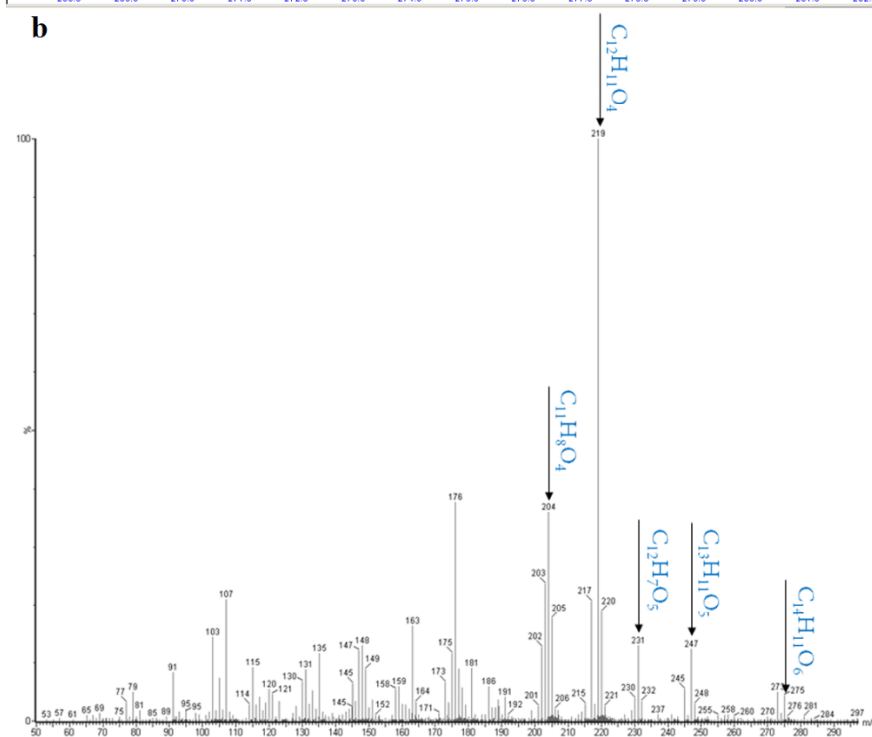
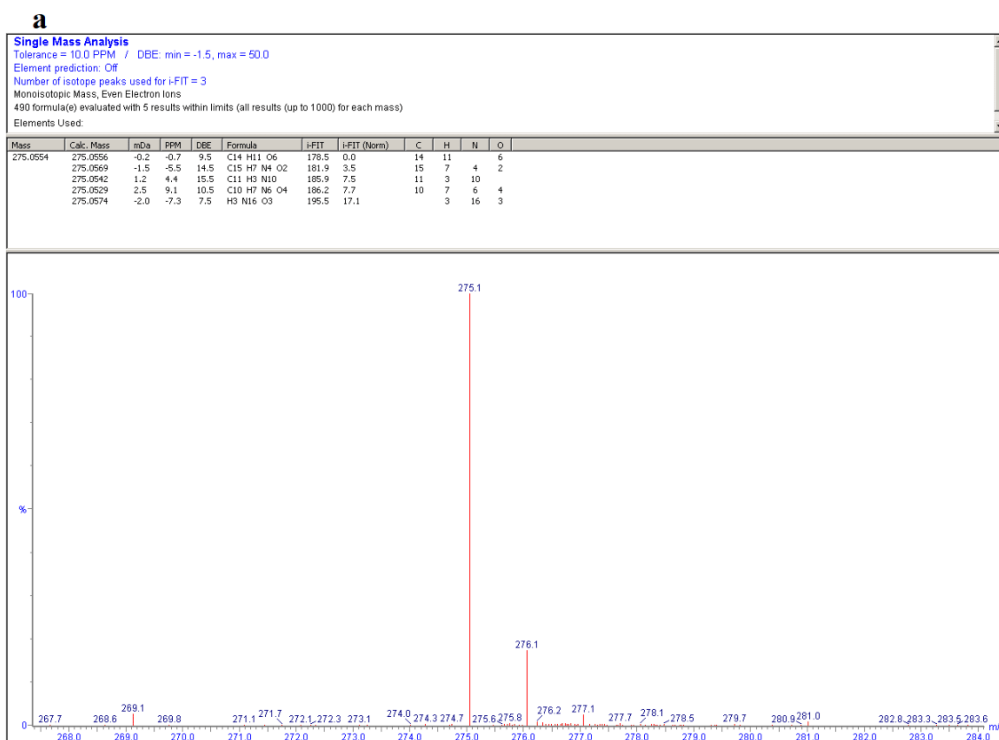
744 observed at m/z 313, 298, 285, 270, 257, 241, 229 and 214.



Supplementary figure 5 | HRMS of P₂. **a** Result of HRMS measurements of $[M+H]^+$ at m/z 299; **b** MS/MS spectrum of parent ion $[M+H]^+$ at m/z 299. Prevalent fragment ions are observed at m/z 271, 255, 243, 228, 215, 200 and 187.

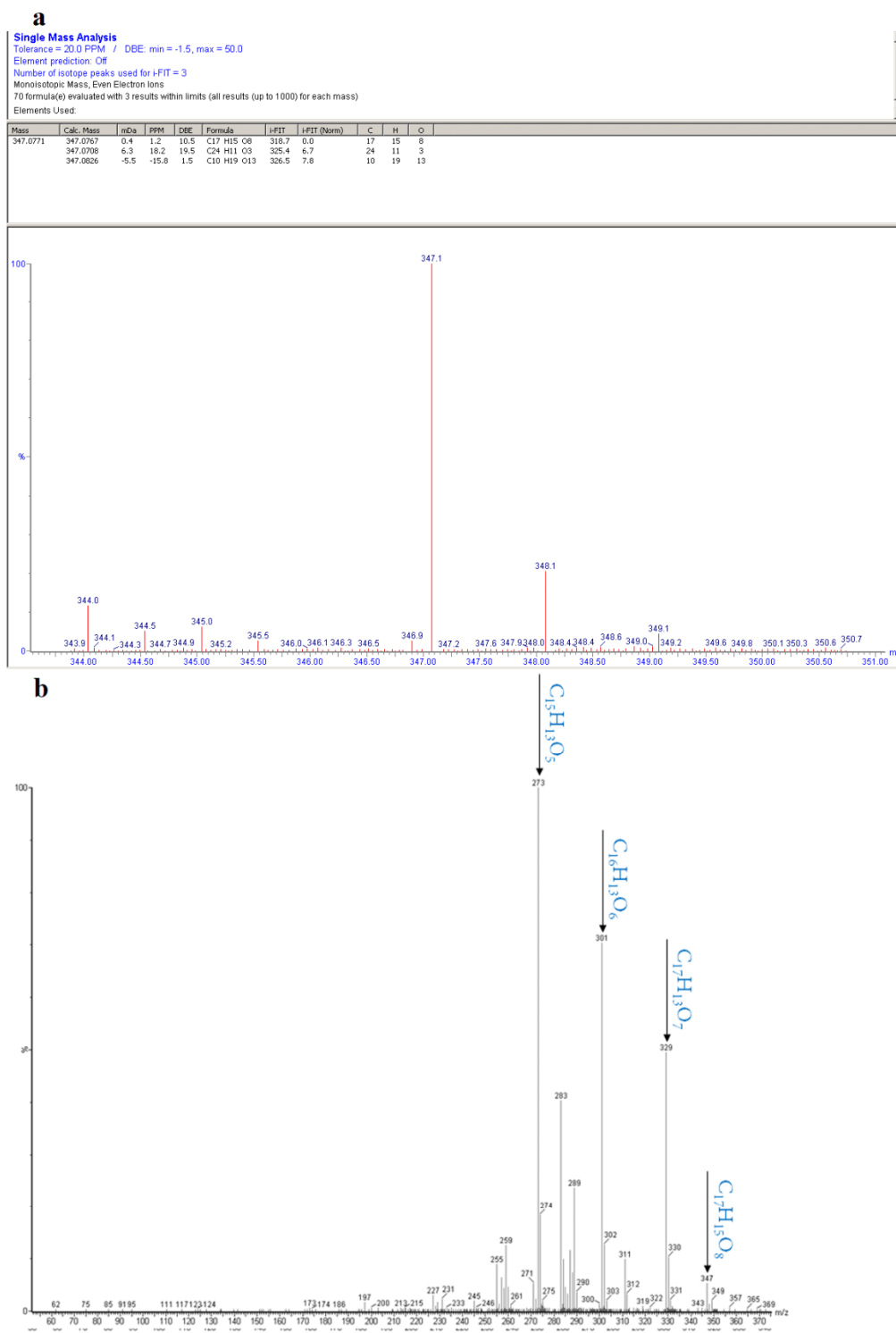


Supplementary figure 6 | HRMS of P3. a Result of HRMS measurements of $[M+H]^+$ at m/z 305; **b** MS/MS spectrum of parent ion: $[M+H]^+$ at m/z 305. Prevalent fragment ions are observed at m/z 287, 259, 244, 231, 216 and 203.



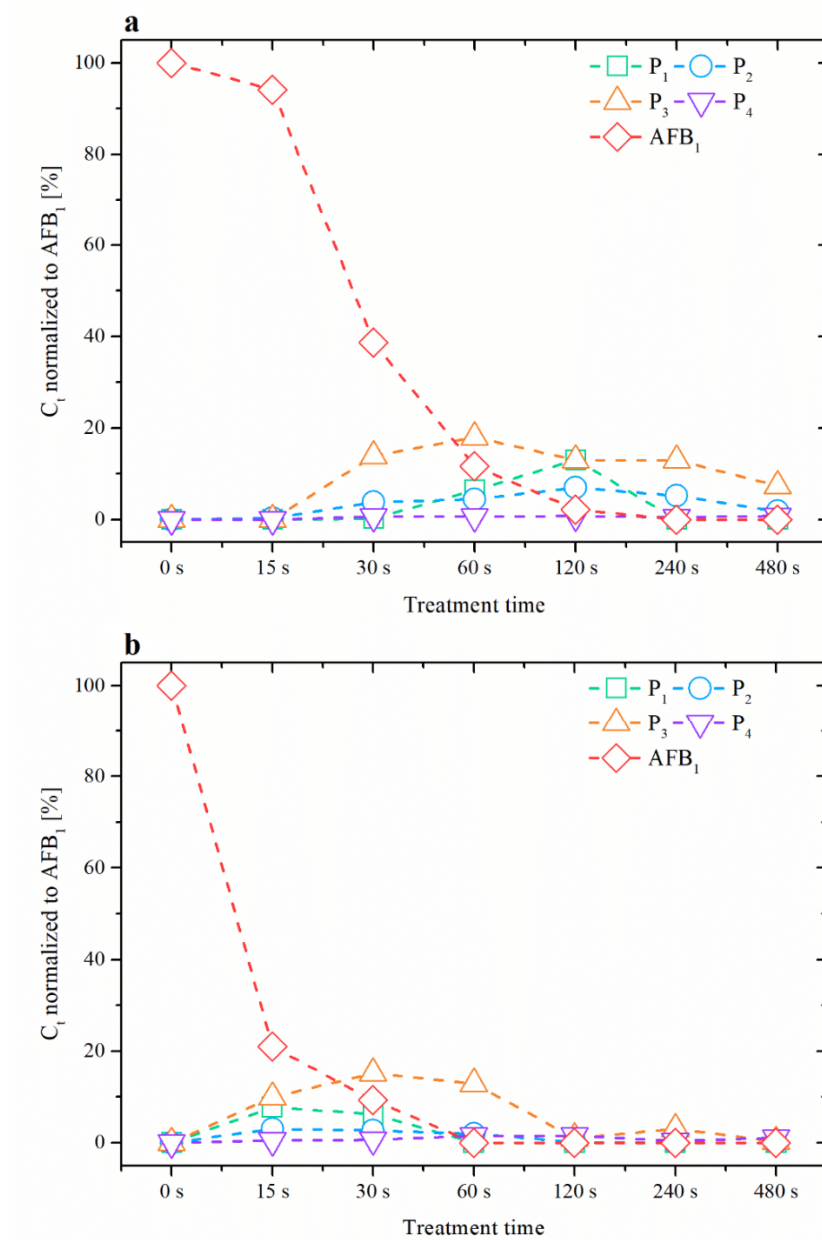
753

754 **Supplementary figure 7 | HRMS of P4. a** Result of HRMS measurements of $[M+H]^+$ at m/z 755 275; **b** MS/MS spectrum of parent ion: $[M+H]^+$ at m/z 275. Prevalent fragment ions are756 observed at m/z 247, 231, 219 and 204.



Supplementary figure 8 | HRMS of p1. a Result of HRMS measurements of $[M+H]^+$ at m/z 347; **b** MS/MS spectrum of parent ion: $[M+H]^+$ at m/z 347. Prevalent fragment ions are observed at m/z 329, 301 and 273.

761 **IV NMR**



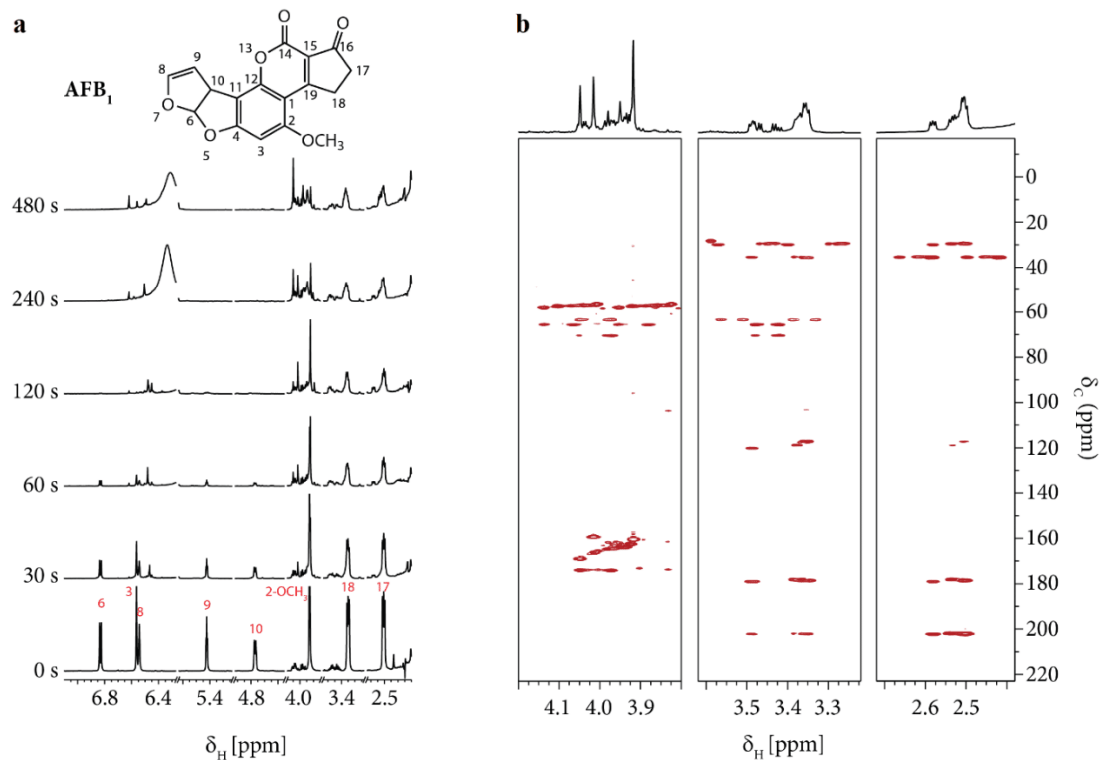
762

763 **Supplementary figure 9 | Degradation of AFB₁ and the formation of degradation products**

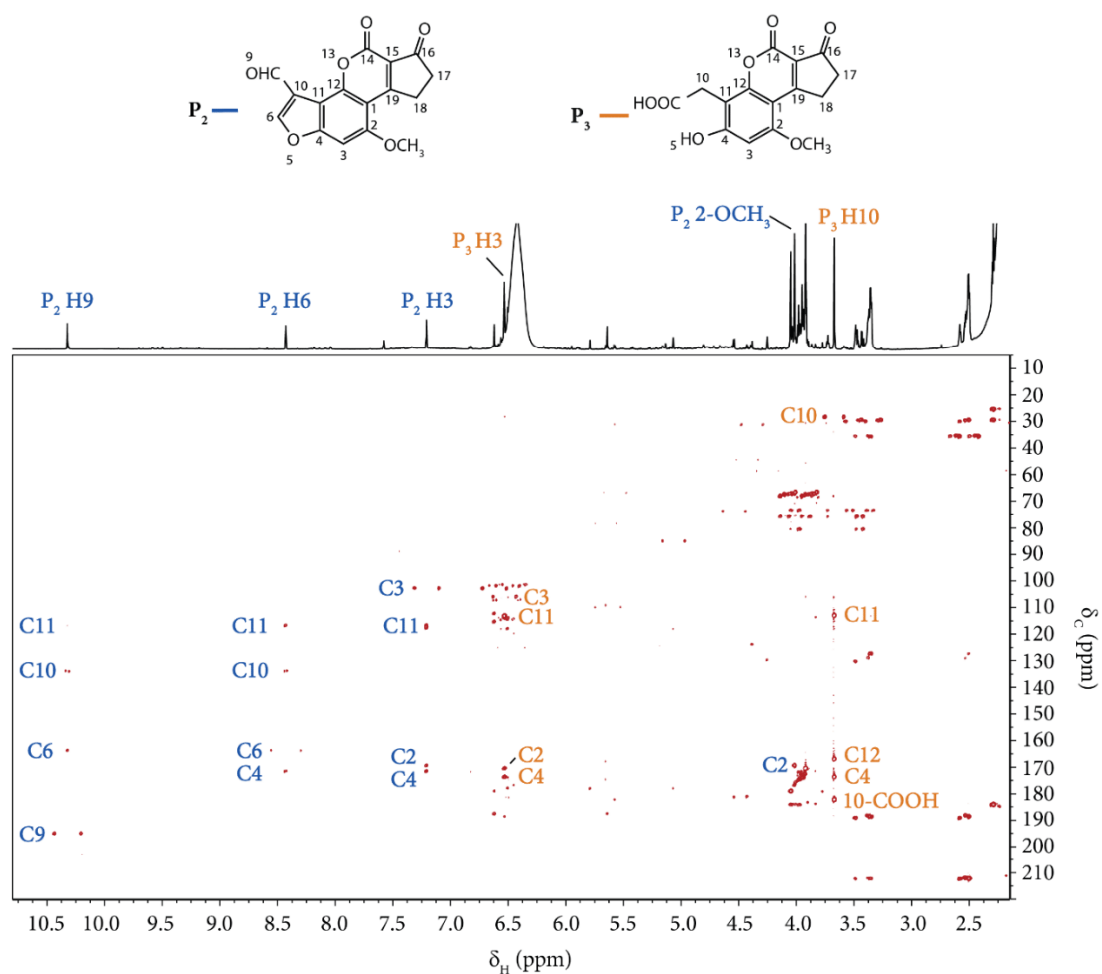
764 **following the intensities of the signals obtained from ¹H NMR.** The concentrations of the

765 degradation products were normalized to the AFB₁ and obtained after the exposure to **a** low

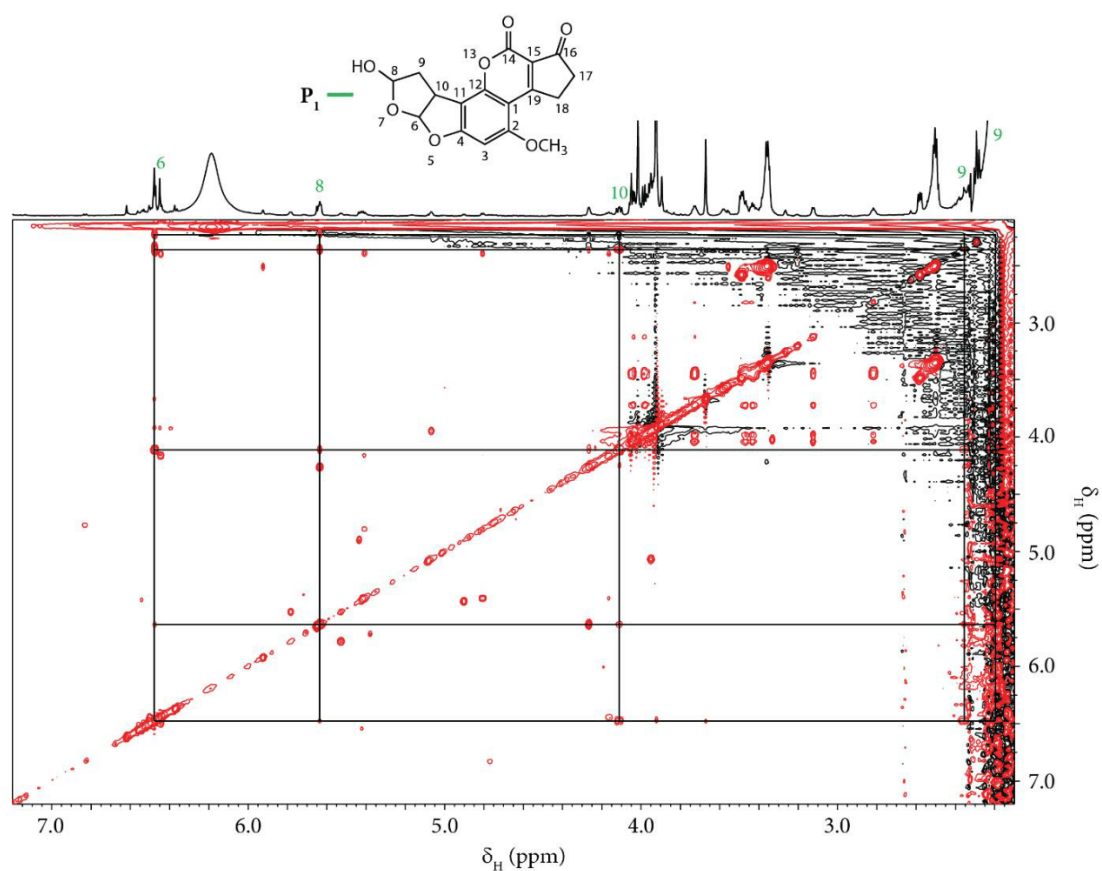
766 and **b** high dose of CAP RONS.



Supplementary figure 10 | **a** Sections of ^1H NMR spectra of AFB₁ sample treated for different time durations with a low dose of CAP RONS showing regions where characteristic signals of AFB₁ can be observed. Signal assignments for AFB₁ are labelled on the spectrum of the non-treated sample (0 s); **b** sections of ^1H - ^{13}C HMBC spectrum of concentrated AFB₁ sample treated with a low dose of CAP RONS for 120 s showing cross-peaks in the ^1H chemical shift ranges characteristic for the 2-methoxy and 17- and 18-methylene groups. The similarity of the observed correlations for the signals belonging to different degradation products indicates that these parts of the molecule are not affected during plasma treatment of AFB₁.



Supplementary figure 11 | ¹H-¹³C HMBC NMR spectrum of AFB₁ sample treated with a low dose of CAP RONS for 120 s in CD₃CN. The assignments for signals of the products **P₂** and **P₃** are labelled in the spectrum. The structures of the products are presented above.

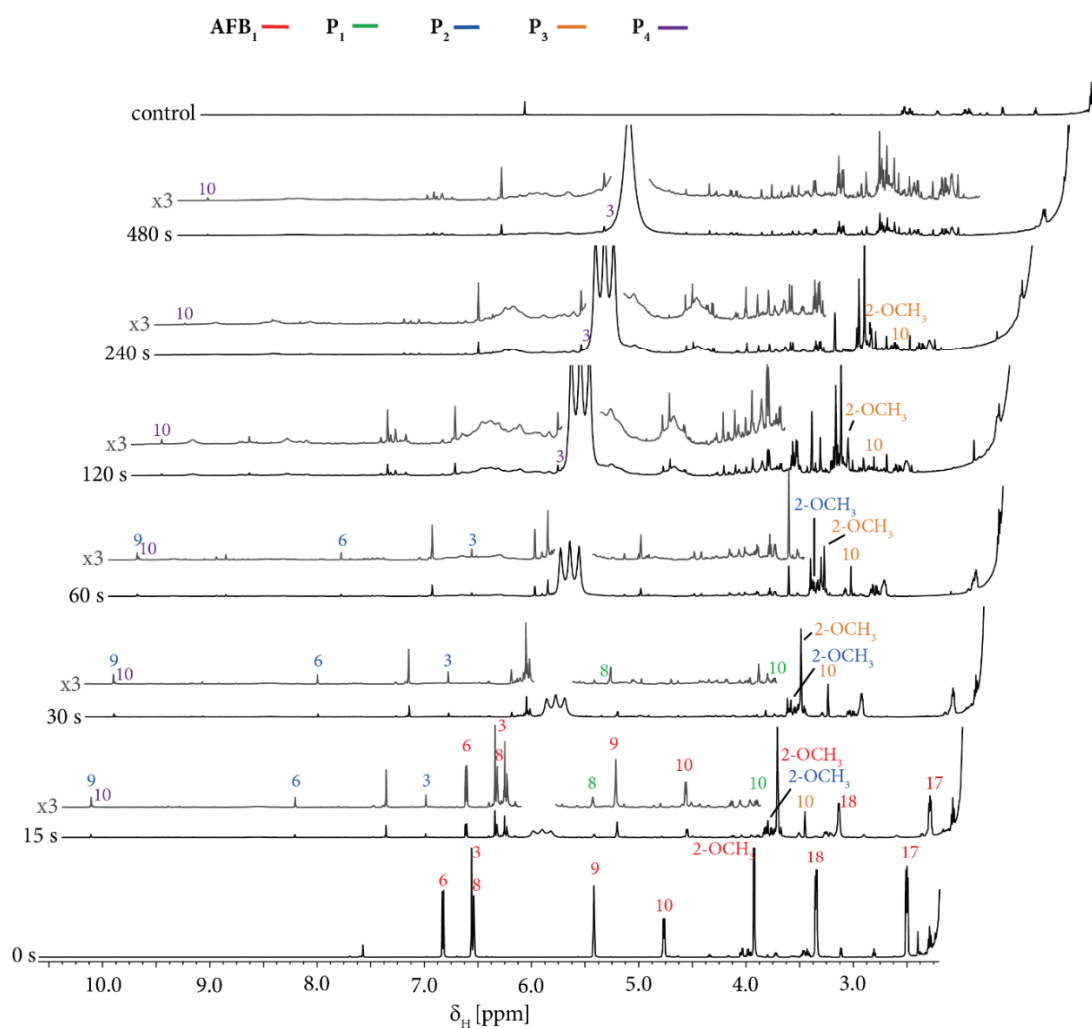


784

785 **Supplementary figure 12 | Section of a TOCSY NMR spectrum of the CAP RONS treated**786 **AFB₁**. The spectrum with 80 milliseconds mixing time was obtained for the AFB₁ sample

787 treated with low dose of CAP RONS for 120 s. The horizontal trace represents the

788 corresponding ¹H NMR spectrum. The spin system assigned to the two furanyl rings of product789 **P₁** is highlighted and the assignments of the protons involved in the system are indicated on the790 ¹H trace.



Supplementary figure 13 | ^1H NMR spectra of AFB_1 samples treated with a high dose of CAP RONS. AFB_1 samples were treated for different time durations and dissolved in the acetonitrile- d_3 . The ^1H NMR spectrum of a control sample (*i.e.* grass coverslip without deposited AFB_1), which was treated with high dose of CAP RONS for 480 s, can be observed at the top.

799 **Supplementary table 1.** List of assigned chemical shifts for identified degradation products
 800 of AFB₁ produced during treatment with CAP RONS.

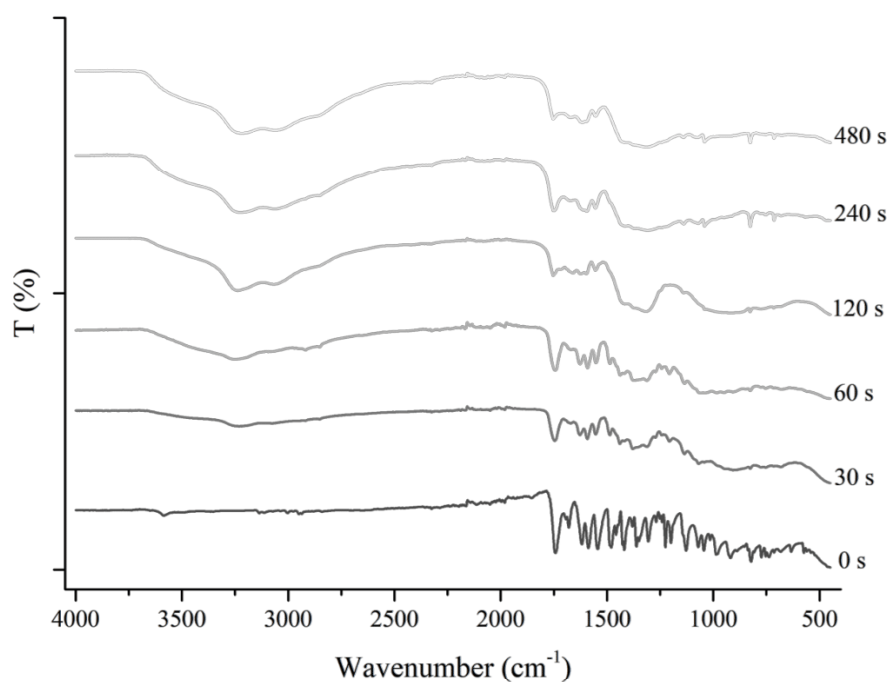
Product	δ_H (ppm)	δ_C (ppm)
P ₁	6.48 (d, J=6.3 Hz, 1H), 6.45 (s, 1H), 5.64 (d, J=5.1 Hz, 1H), 4.11 (dd, J=9.5, 6.3 Hz, 1H), 3.92 (s, 3H), 3.39-3.31 (m, 2H), 2.55-2.46 (m, 2H), 2.36 (ddd, J=13.4, 9.7, 5.2 Hz, 1H), 2.22 (m, 1H).	166 (C4), 162 (C2), 114 (C6), 109 (C11), 103 (C1), 100 (C8), 90 (C3), 56 (2-OCH ₃), 42 (C10), 37 (C9).
P ₂	10.32 (s, 1H), 8.43 (s, 1H), 7.21 (s, 1H), 4.02 (s, 3H), 3.51-3.47 (m, 2H), 2.60-2.56 (m, 2H).	185 (C9), 161 (C4), 159 (C2), 154 (C6), 124 (C10), 107 (C11), 107 (C1), 93 (C3), 58 (2-OCH ₃).
P ₃	6.53 (s, 1H), 3.92 (s, 3H), 3.67 (s, 2H), 3.48-3.50 (m, 2H), 3.39-3.31 (m, 2H), 2.55-2.46 (m, 2H).	203 (C16), 178 (C19), 173 (10-COOH), 164 (C4), 161 (C2), 157 (C12), 120 (C15), 104 (C11), 96 (C3), 57 (2-OCH ₃), 36 (C17), 30 (C18), 29 (C10).
P ₄	10.32 (s, 1H), 6.52 (s, 1H), 3.39-3.31 (m, 2H), 2.55-2.46 (m, 2H).	^{b)}

801

802 a) The signal is overlapped by the water signal.

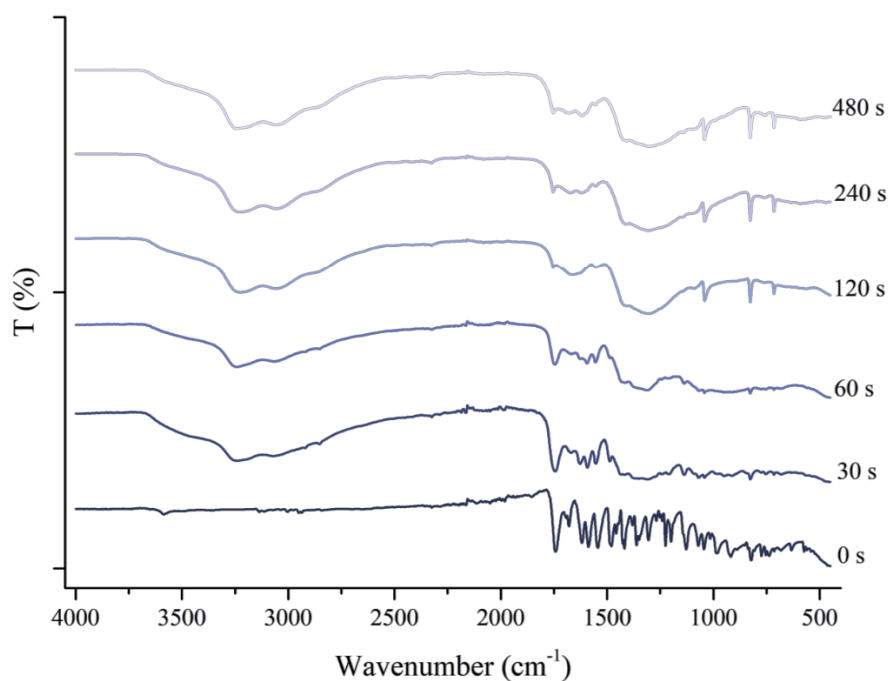
803 b) P₄ is a low concentration degradation product, which did not permit detection of ¹³C NMR.

804

805 **V FT-IR of CAP RONS treated AFB₁**

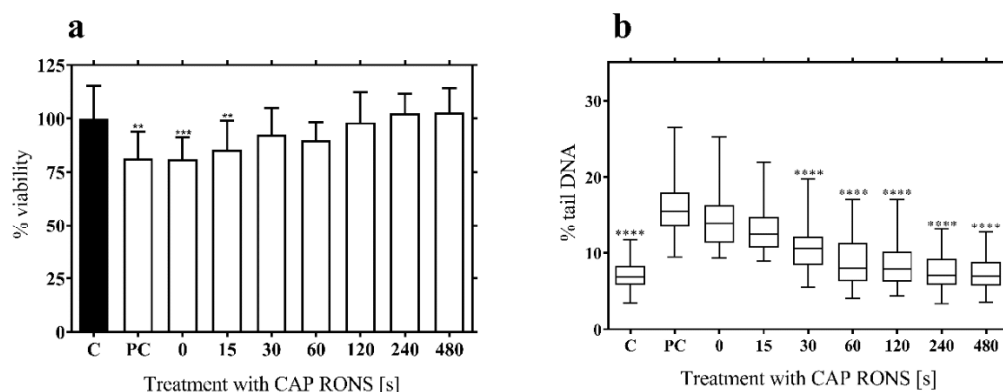
806

807 **Supplementary Figure 14 | FT-IR spectra of CAP RONS treated AFB₁.** The spectra were
808 obtained from the AFB₁ samples following their exposure to a low dose of CAP RONS for
809 different time durations.



Supplementary Figure 15 | FT-IR spectra of CAP RONS treated AFB₁. The spectra were obtained from the AFB₁ samples following their exposure to a high dose of CAP RONS for different time durations.

824 VI Toxicity tests



825

826 Supplementary figure 16 | Reduction in AFB₁ toxicity following exposure to plasma

827 **generated RONS.** AFB₁ exposed to low dose of CAP generated RONS was applied to HepG2

828 cells for 24 hours. Controls were left unexposed. The positive control contained 30 μ M of

829 AFB₁; **a** Cell viability was determined using an MTS assay. Three independent experiments,

830 each containing five replicates, were performed. The results are presented as mean values $\pm$

831 SD. *P < 0.01; **P < 0.001 ***P < 0.0001 (one-way ANOVA, Dunnet post-tests); **b** The

832 generation of DNA strand breaks after the exposure to treated AFB₁ was examined using the

833 comet assay, and is presented as percentages of DNA in the comet tails. The distribution of the

834 data is presented in box-plots. Three independent experiments were performed with fifty cells

835 analysed in each experimental point. *P < 0.01; **P < 0.001 ***P < 0.0001 (two-way ANOVA,

836 Dunnet post-tests)

837

838

839 **VII Identification of the products**840 **AFB₁**

841 ¹H NMR (600 MHz, CD₃CN): δ 6.83 (d, J=7.0 Hz, 1H), 6.56 (s, 1H), 6.54 (t, J=2.5 Hz, 1H), 5.42 (t,
842 J=2.5 Hz, 1H), 4.77 (dt, J=7.0, 2.5 Hz, 1H), 3.93 (s, 3H), 3.39-3.31 (m, 2H), 2.55-2.46 (m, 2H); ¹³C
843 NMR (151 MHz, CD₃CN): δ 202.3, 178.7, 166.2, 162.6, 155.8, 153.6, 146.2, 114.7, 108.3, 104.8, 103.5,
844 91.9, 57.5, 48.5, 35.8, 29.7; HRMS (ESI) m/z: [M+H]⁺ calculated for C₁₇H₁₃O₆ 313.0712; found
845 313.0710

846 **P₁**

847 ¹H NMR (600 MHz, CD₃CN): δ 6.48 (d, J=6.3 Hz, 1H), 6.45 (s, 1H), 5.64 (d, J=5.1 Hz, 1H), 4.11 (dd,
848 J=9.5, 6.3 Hz, 1H), 3.92 (s, 3H), 3.39-3.31 (m, 2H), 2.55-2.46 (m, 2H), 2.36 (ddd, J=13.4, 9.7, 5.2 Hz,
849 1H), 2.22 (m, 1H); HRMS (ESI) m/z: [M+H]⁺ calculated for C₁₇H₁₅O₇ 331.0818; found 331.0815.

850 **P₂**

851 ¹H NMR (600 MHz, CD₃CN): δ 10.32 (s, 1H), 8.43 (s, 1H), 7.21 (s, 1H), 4.02 (s, 3H), 3.51-3.47 (m,
852 2H), 2.60-2.56 (m, 2H); HRMS (ESI) m/z: [M+H]⁺ calculated for C₁₆H₁₁O₆ 299.0556; found
853 299.0558.

854 **P₃**

855 ¹H NMR (600 MHz, CD₃CN): δ 6.53 (s, 1H), 3.92 (s, 3H), 3.67 (s, 2H), 3.48-3.50 (m, 2H), 3.39-3.31
856 (m, 2H), 2.55-2.46 (m, 2H); HRMS (ESI) m/z: [M+H]⁺ calculated for C₁₅H₁₃O₇ 305.0661; found
857 305.0669.

858 **P₄**

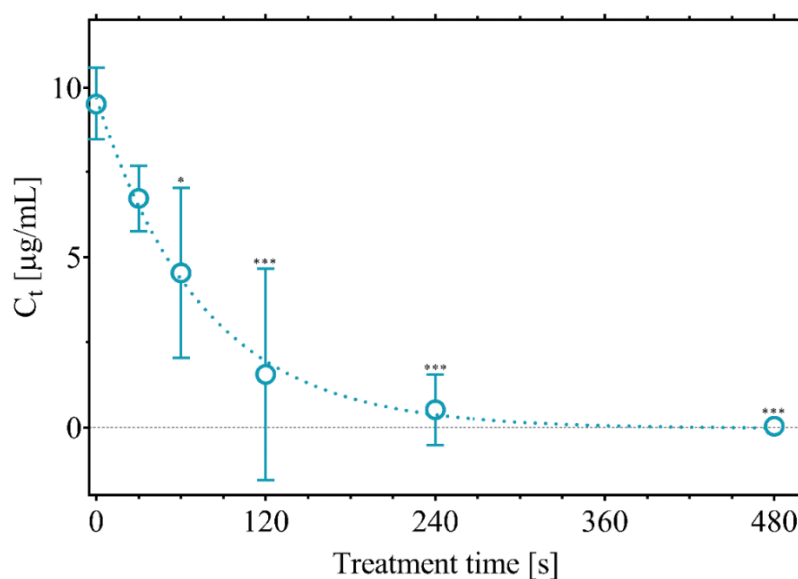
859 ¹H NMR (600 MHz, , CD₃CN): δ 10.32 (s, 1H), 6.52 (s, 1H), 3.39-3.31 (m, 2H), 2.55-2.46 (m, 2H);
860 HRMS (ESI) m/z: [M+H]⁺ calculated for C₁₄H₁₁O₆ 275.0556; found 275.0554

861 **p₁**

862 HRMS (ESI) m/z: [M+H]⁺ calculated for C₁₇H₁₅O₈ 347.0767; found 347.0771.

863

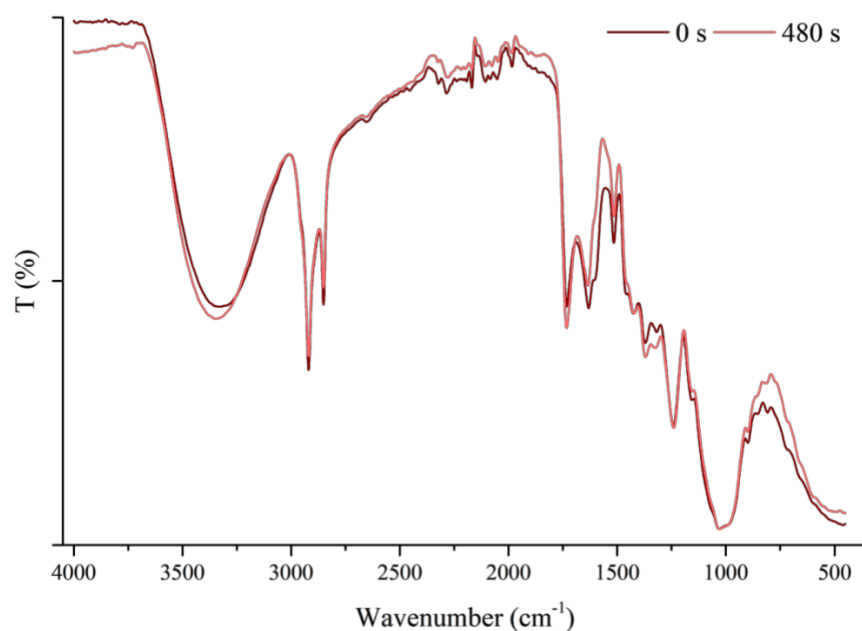
VII Preliminary results of AFB₁ decontamination with CAP RONS based
on real case (corn)



866

867 **Supplementary figure 17 | AFB₁ decontamination efficiency based on CAP RONS**
 868 **treatment of artificially contaminated corn.** Contaminated corn kernels were exposed to
 869 CAP RONS, using the same experimental parameters as in the case of treatment of AFB₁
 870 samples placed on glass coverslips. The experiments were performed in multiple replicates and
 871 are presented in mean values $\pm$ SD. *P < 0.01; **P < 0.001 ***P < 0.0001 (one-way ANOVA,
 872 Dunnet post-tests; n=3).

873



874

875 **Supplementary figure 18 | FT-IR spectrum of outer corn layer.** The spectrum was obtained
 876 after the exposure to CAP RONS for 480 seconds. No difference was observed after the
 877 comparison to the spectrum of a non-treated sample (0 s).

878

879

Chapter 6

Conclusions and Future Outlook

The experimental results presented in this thesis demonstrated that non-equilibrium CAP technology is suitable for the decontamination of mycotoxins and inactivation of mycotoxigenic fungi. Moreover, the efficacy of CAP based on the air SBD system was significantly higher when compared to the competitive decontamination and disinfection methods. The high performance of CAP was attributed to the oxidative properties, and high concentrations of CAP-generated RONS. The latter mediated the decay of AFB₁ leading to its complete degradation or the formation of degradation products which lost the toxicity of the parent mycotoxin molecule.

Further discussion regarding results is concluded by each stated objective:

Objective 1: To optimise the treatment process to obtain the most efficient CAP conditions for decontamination of mycotoxins and inactivation of fungi

Various mycotoxins and *A. flavus* were exposed to CAP under different applied voltages, creating different discharge chemistries, which contained different concentrations of RONS. The treatments were performed in different time regimes, which also influenced the dynamics of the formation of RONS. The most significant efficacies were obtained after the treatment with CAP generated with high applied voltages. The most efficient mycotoxin removal was observed for the treatment of AFB₁, AFG₁ and ZEN where more than 99% decontamination rate was achieved in only 60 s of treatment. When compared to common food processing methods such as UV-C irradiation and thermal treatment, CAP performance of the decontamination performance was of magnitude higher.

In the case of inactivation of *A. flavus* spores, a 5 log reduction was obtained after 120 s of direct exposure to CAP gaseous discharge. No disinfection activity was observed even after 8 min of treatment with standard 1% Virkon® disinfectant. In contrast, Virkon® was significantly more efficient in the indirect treatments, where PAB obtained after the treatment with high power CAP for 8 minutes induced only 1 log reduction in newly grown spores.

The objective was achieved in Chapters 2, 3, 4 and 5.

Objective 2: To characterise the plasma discharge chemistry and key RONS in the gaseous and liquid phase of discharge

Different applied voltages were used to ionise, excite and dissociate the gaseous molecules in ambient air. SBD plasma systems used for the experiments thesis were operated at three different applied voltages, creating three different RONS chemistries, which were analysed with FTIR and OES. At low applied electrical power input, low concentrations of RONS were created, with ROS such as O₃ as prevailing species. A high applied voltage induced the formation of high concentrations of RONS. The poisoning of O₃ was observed, which was followed by an intense rise of the RNS (i.e. NxOy). A medium applied voltage generated the chemistry with concentrations of RONS reaching the values between low and high power discharge conditions. Considering the distance gap between the electrode and the treated sample, CAP decontamination and inactivation processes were dictated by long-lived RONS.

Plasma-treated liquid was characterised with the emphasis on the determination of pH and the concentrations of H₂O₂, NO₂⁻ and HNO₃. As in the case of plasma gaseous effluent, the plasma-induced modifications of liquid chemistry were largely influenced by the applied voltages and

discharge conditions. In addition, plasma liquid chemistry also depended on the type of liquid and the liquid volume. HNO_3 was the prevalent species in all three types of RONS chemistries, with the highest concentrations observed after the long exposures of aqueous broth solution to high discharge power conditions. This induced a significant drop in pH, resulting in the fast transformation of H_2O_2 and NO_2^- into ONOO^- and later NO_3^- , which can be observed at the sudden drop of their concentrations. This reaction typically occurred when the pH levels decrease under 4.

The objective was achieved in Chapters 3 and 5.

Objective 3: To identify the most important degradation products generated after the exposure of mycotoxins to RONS and to reveal the underlying degradation mechanisms

Following AFB_1 exposure to CAP-generated RONS, four key degradation products were identified. These products were observed in all plasma discharge conditions. Given that the CAP-induced RONS chemistry was defined, RONS-induced decontamination mechanisms of AFB_1 were determined. The degradation started at the double bond between C8 and C9 and then divided into multiple pathways. When one molecule of water was added to AFB_1 , 8-hydroxyd AFB_1 derivate (P_1 ; Aflatoxin B_{2a}) was formed. Immediately after, the new stable aromatic product 9-methoxy-5,6-dioxo-5,6,7,8-tetrahydrocyclopenta[c]furo[2,3-h]chromene-3-carbaldehyde (P_2) was generated through the radical reaction involving OH and both AFB_1 and P_1 . Alternatively, peroxides induced the formation of AFB_1 -8,9-exo epoxide, at given conditions highly unstable product, which was spontaneously hydrolysed into product AFB_1 -8,9-diol (p_1), also produced in the reaction of P_1 with OH. With the reversible conversion to its hydroxydialdehyde form, CAP RONS were able to attack inner atoms of difuranyl part of AFB_1 , eventually resulting in the formation of product 2-(7-hydroxy-9-methoxy-3,4-dioxo-1,2,3,4-tetrahydrocyclopenta[c]chromen-6-yl)acetic acid (P_3). Additional removal of carboxylic group from P_3 or the cleavages of C6, C8 and C9 from p_1 led to the generation of another aromatic aldehyde product 7-hydroxy-9-methoxy-3,4-dioxo-1,2,3,4-tetrahydrocyclopenta[c]chromene-6-carbaldehyde (P_4) as a terminal degradation product in the CAP RONS degradation of AFB_1 .

The objective was achieved in Chapter 5.

Objective 4: To determine the potential toxicity of degradation products

The systematic experimental approach presented in this thesis was concluded with the toxicological tests, which provided data about the toxicity of the degraded AFB_1 , achieved after the exposure to CAP. An absolute need of any decontamination study such as ours is a double-test toxicological examination, which demonstrated that only a 15 s treatment of AFB_1 at a high dose of CAP RONS is required to increase the viability of Hep G2 cells to the levels comparable with controls. In contrast, the number of DNA single-strand breaks is reduced to normal values only after 60 s. This uncovers once more the urgency of the thorough toxicological investigation, which involves the selection of the right tests that provide the accurate information about the potential toxicity of the tested compounds.

The objective was achieved in Chapter 5.

Conclusions and propositions for the future

Considering the discussion, this thesis hypothesised that CAP technology is suitable for the decontamination of different types of mycotoxins. Given that CAP completely removed the mycotoxins or reduced their concentrations under the maximum permissible levels in the selected food products, our hypothesis on CAP decontamination efficacy was partially confirmed. The preliminary results obtained from the CAP treatment of AFB_1 -contaminated corn indicated that the chemical structure of the corn kernel surface remains intact.

We assumed that CAP treatment also inactivates *A. flavus*, which was confirmed in the case of the direct exposure employing the plasma gaseous RONS rich effluent. Conversely, the results demonstrated that indirect treatment with PAB was not an applicable approach for mould disinfection.

The last hypothesis focused on the CAP-promoted AFB₁ degradation mechanisms. It was proven that RONS-based plasma chemistry induced the decay of an AFB₁ molecule, leading to the formation of new products with an altered chemical structure. The toxicological tests demonstrated that CAP exposures resulted in elimination of the AFB₁ toxicity. Nevertheless, AFB₁ metabolites AFB_{2a} and AFB₁-8,9-diol were also identified as CAP-induced AFB₁ degradation products, which are known for their cytotoxicity. The absence of toxic effects following the AFB₁ exposure to CAP was therefore also attributed to the further decay of degradation products.

This thesis offers an insight into the comprehensive research which was performed with the aim to define the fundamentals of non-equilibrium CAP as a new mycotoxin decontamination method. The experiments performed in the thesis will serve as a case study for future investigations in the field of plasma decontamination of other toxic compounds. Nevertheless, Chapter 4 stressed that the decontamination efficiencies varied from one type of mycotoxin to another. This indicates that CAP decontamination is also influenced by the chemical structure of a specific mycotoxin. Therefore, a detailed examination of the CAP-induced degradation mechanisms is necessary, involving not only AFB₁ but also other types of mycotoxins. In addition, the impact of RONS on the food matrix properties needs to be evaluated fully. For any novel food processing technology to be accepted, the influence of the procedure on the nutritional, safety and organoleptic properties of the treated food must be fully characterised.

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Biography

The author of this work graduated from Biotechnology at the Biotechnical Faculty, University of Ljubljana, Slovenia, with the thesis entitled »The effect of insecticide coumaphos on behaviour of laboratory mice as a model for human toxicity« under the supervision of Prof. Dr. Gregor Majdič. In 2011, she started working at PKG, Šprinzer Mirko s.p., where she was involved in European LIFE projects, including Rebirth, which was selected as one of the best European environmental projects in 2015. In 2014, she started working at the Jožef Stefan Institute (JSI) as a young researcher and started her doctoral studies at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia. In the last four years, she mostly worked with mycotoxins and their removal with atmospheric pressure plasma technology and was trained in the techniques such as UV-VIS spectrometry, LC-MS, FTIR and basic microbiological and toxicological tests.

During her employment at the JSI, she also collaborated with other leading Slovenian institutes, including the National Institute of Chemistry, the National Institute of Biology, the Institute of Oncology, and the Institute for Hygiene and Pathology of Animal Nutrition. She presented her results at a number of different internationally recognised conferences and symposiums. In addition, she finished a one-month long internship ("short-term scientific mission – STSM) at the University of Liverpool, United Kingdom.