Public health aspects of *Fusarium* mycotoxins in food in The Netherlands

A risk assessment

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Voor mijn ouders

STELLINGEN

1 De onderzoeken uitgevoerd door Braak *et al.* (1997) en Schouten *et al.* (1997) om de overdracht van antibioticaresistente micro-organismen van dier naar mens te bewijzen schieten in experimentele opzet te kort om conclusies te trekken.
van den Braak., N., Kreft, D., van Belkum., A., Verburgh, H., Endtz, H. (1997) The Lancet ***350,*** *146-147.*
Schouten, M. A., Voss, A., Hoogkamp-Korstanje, J. A. A. (1997) The Lancet, ***349,*** *1258.*

2 Kiemgetallen hoger dan de wettelijke norm kunnen worden aangetroffen in UHT verhitte melk als gevolg van besmetting met zeer hitteresistente bacteriële sporenvormers. Gesteriliseerde levensmiddelen zijn dus niet altijd steriel.
Hammer, P., Lembke, F., Suhren, G. en Heeschen, W. (1995) Kieler Milchwissenschaftliche Forschungsberichte ***47,*** *297-305.*
Pettersson, B., Lembke, F., Hammer, P., Stackebrandt, E. and Priest, F. G. (1996) Int. J. System. Bacteriol. ***46,*** *759-764.*

3 De conclusie van Bentham en Langford (1995) dat het aantal voedselinfecties over de afgelopen 15 jaar jaarlijks is toegenomen als gevolg van het broeikast effect, gaat voorbij aan het feit dat registratie van voedselinfecties en opslag in databanken van deze gegevens in die periode tot ontwikkeling is gekomen.
Bentham, G. en Langford, I. H. (1995) Int. J. Biometeorol. ***39,*** *81-86.*

4 Het is een hardnekkig misverstand in de levensmiddelenmicrobiologische literatuur dat reducerende suikers en sucrose in levensmiddelen een redoxpotentiaal-verlagende werking zouden hebben.
FLAIR Concerted Action no 7, subgroup B. (1994) EUR 15776 EN.

5 Bij de analyse van bacteriën met pyrolyse massaspectrometrie kan het discriminerend vermogen sterk worden verbeterd door de meting van hoger moleculaire verbindingen, zoals di- en triglyceriden, die dragers zijn van belangrijke biochemische informatie.
Tas, A. C. en van der Greef, J. (1994) Mass Spectrometry Rev. ***13,*** *155-181.*

6 Daar aflatoxine wordt beschouwd als te gebruiken gifstof in biologische wapens kunnen levensmiddelen die besmet zijn met dit mycotoxine beschouwd worden als biologische wapens. Het zou dan ook overwogen moeten worden om, naast het ministerie van volksgezondheid, welzijn en sport, het ministerie van defensie te betrekken bij de normstelling en handhaving inzake dit mycotoxine.
Bionieuws `Dodend leven, micro-organismen als massa-vernietigingswapen`, 21 juni, 1997.
de Volkskrant `Pistachehandel gevraagd om controle voorraden op gifstof`, 11 september, 1997.

7 `Natuurlijk` in het begrip `natuurlijk toxine` heeft een tegengestelde betekenis aan `natuurlijk` in het begrip `natuurlijke smaakstof`.

8 Het is mogelijk dat een deel van de `erfelijke kanker` verklaard kan worden uit het feit dat alle leden van een gezin hetzelfde voedsel nuttigen en dus blootstaan aan de dezelfde daarin voorkomende carcinogenen.

9 Zearalenon in brood kan de blootstelling van mensen aan (pseudo-) oestrogenen verhogen en bijdragen aan de verminderde vruchtbaarheid van de man.

10 Het puntig kaalkopje (*Psilocybe semilanceata,* halucinogene paddo) leidt tot hoofdbrekens bij de wetgever.
de Volkskrant `Kaalkopjes onschuld`, 18 januari, 1997.

11 Het ziekteverzuim als gevolg van luchtweginfecties zou verlaagd kunnen worden als deelnemers aan carpools ingedeeld werden op grond van het wel of niet hebben van kinderen die naar crèche, peuterspeelzaal of school gaan.

Stellingen behorende bij het proefschrift:

Public health aspects of
***Fusarium* mycotoxins in food**
in The Netherlands
A risk assessment

Monique de Nijs

Wageningen, 13 januari 1998

ABSTRACT

Monique de Nijs (1997) *Public health aspects of* Fusarium *mycotoxins in food in The Netherlands:* A risk assessment. PhD-thesis, Wageningen Agricultural University, The Netherlands (140 p., summaries in English and Netherlands).

Plant pathogenic *Fusarium* moulds occur world-wide and cereals can become infected during the growing period. *Fusarium* was detected in 83 % of 69 cereal samples of batches intended for food or feed production and harvested in The Netherlands in 1993. A considerable genotypic and phenotypic variation was observed within two of the most frequently isolated *Fusarium* species. Mycotoxins can be excreted in the crop by the fungus after the plant becomes infected. A literature review revealed 137 secondary metabolites that could be produced by *Fusarium* species which were isolated from food raw materials. Twelve of those secondary metabolites were identified as mycotoxins based on toxicity observed in test animals. Six of those twelve have possibly been involved in human disease outbreaks (T-2 toxin, nivalenol, deoxynivalenol, acetyldeoxynivalenol, fumonisin B_1 and zearalenone). Most of the mycotoxins are stable under process conditions used for food production and can be detected in food. Cereals harvested in The Netherlands in 1993 were contaminated with deoxynivalenol (food poisoning and immunotoxic), 3 %, or zearalenone (oestrogen), 1 %. Fumonisin B_1 (carcinogenic, related to human oesophageal cancer) was detected in 98 % of samples of maize from batches imported in The Netherlands and intended for food production. A 28-day toxicity study on the effects of fumonisin B_1 in rats revealed dose-response related apoptosis in the kidney. The lowest observed effect level was at 0.19 mg fumonisin B_1 kg^{-1} rat body weight. The data on fumonisin B_1 toxicity were used to estimate a tolerable daily intake (TDI) of 500 ng fumonisin B_1 kg^{-1} human body weight. The probability of being daily exposed to fumonisin B_1 at a level corresponding to this TDI was 12 % for the people in The Netherlands consuming the average amount of maize, 55 % for people belonging to the group of `eaters only` and 78 % for people with gluten intolerance. The health of the consumers in The Netherlands might, in the current situation, be challenged by *Fusarium* mycotoxins present in food. Deoxynivalenol, which has immunotoxic characteristics, can potentially be present in food and feed and might increase human exposure to infectious diseases, especially to those from zoonotic origin.

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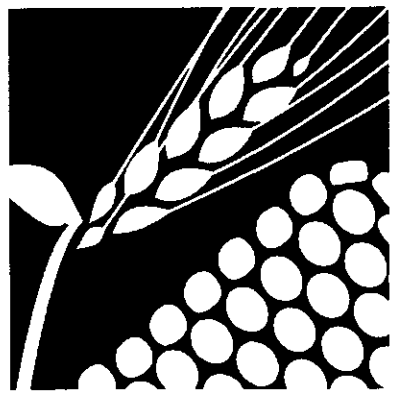
General introduction



BACKGROUND

Increasing attention is currently paid to mycotoxins, toxic secondary metabolites produced by moulds, that can be present in food. These natural toxins can adversely affect the health of humans and animals. Fungi can excrete the mycotoxins in the plant when the crops are infected in the field or after harvest when crops are transported or stored incorrectly. The mycotoxins can be transferred to food or feed when the mycotoxins can resist the processes used for preparation of the commodities. Carry-over of mycotoxins from feed through animal products on humans may occur. In this thesis, a risk assessment is carried out on mycotoxins produced by species of the fungal genus *Fusarium*. Risk assessment of *Fusarium* mycotoxins in foods is required for risk management to guarantee consumption of acceptable safe food with regard to *Fusarium* mycotoxins.

FUSARIUM INFECTION AND MYCOTOXIN PRODUCTION

Fusarium is the most important field pathogen of crops world-wide and the fungus can be detected in nearly all food crops *(47)*. When considering *Fusarium* mycotoxin contamination of food raw materials, cereals are the most important group. Most

work on toxic effects of *Fusarium* mycotoxins dealt with farm animals receiving cereal based diets. Carry-over of *Fusarium* mycotoxins from feed through animal products (meat or milk) on humans is currently not known *(12, 26)*.

Cereal crops are infected with *Fusarium* species in the field. *F. graminearum* and *F. culmorum* are plant pathogenic fungi, invading the plant, while *F. moniliforme* infects senescent or stressed plants *(40)*. Infection with *Fusarium* can be symptom-free and viable mycelium and spores can be present, endogenous, in seeds at harvest *(34, 69)*.

The genetic characteristics of the *Fusarium* species and strains present in the crop and the environmental factors influencing mycotoxin production by the *Fusarium* moulds present, determine the contamination of food raw materials with *Fusarium* mycotoxins. The production of mycotoxins can be restricted to certain *Fusarium* species, as in case of fumonisin B_1, or to individual *Fusarium* isolates within the species *(16, 45, 56)*. Environmental factors, such as climate, influence *Fusarium* infection in the crop and may favour the occurrence of certain *Fusarium* species or isolates and, thus, influence mycotoxin production *(2, 11, 18, 32, 44, 45, 66, 75)*. Agricultural practices, such as the use of fungicides, might reduce *Fusarium* infection and can thus reduce the mycotoxin contamination of cereals *(10, 28)*. Several fungicides are not effective against *Fusarium* and resistance of *Fusarium* species to fungicides is reported *(27, 35, 78)*. Several authors report on increase of mycotoxin production by *Fusarium* due to the use of fungicides *(22, 42)*. The application of fertilisers and the use of susceptible cereal cultivars can increase *Fusarium* infection and thus increase the risk of mycotoxin contamination of cereals *(20, 54)*. In addition, humidity and environmental temperature influence mycotoxin production by the fungus *(1, 25, 37)*. Though mycotoxin production by *Fusarium* appears to be limited to the field period, the production of mycotoxins by endogenous viable mycelium can not be excluded when cereals are transported or stored under poor conditions after harvest *(34)*.

FUSARIUM SECONDARY METABOLITES AND SYNTHESIS

Species of the genus *Fusarium* can produce over 130 secondary metabolites *(46)*.

The largest group of secondary metabolites produced by *Fusarium* are the trichothecenes which can be subdivided into type A and type B. Trichothecenes belonging to type B are characterised by a ketone group on C_8 (Fig. 1) and are less toxic than trichothecenes belonging to type A *(67)*. Most of the type B trichothecenes can be determined by HPLC with fluorescent detection as opposed to the type A trichothecenes. The more complicated methods required for the detection of trichothecenes type A may explain the lower number of reports on the natural occurrence of these mycotoxins. Several metabolites of the trichothecenes, e.g., deoxynivelenol (Fig. 1), have known adverse effects on the health of humans and farm animals. Other groups of secondary metabolites produced by *Fusarium* with known toxic effects on animal and, allegedly, on human health are the zearalenones and fumonisins *(46)*.

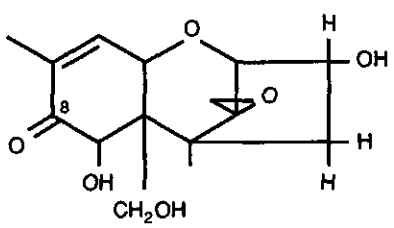


Figure 1. *Molecular structure of deoxynivalenol (trichothecene type B).*

Secondary metabolites are synthesised in successive steps which are catalysed by enzymes *(41, 80, 81)*. Most information is available on the synthesis of trichothecenes. The key enzyme in the first step of the synthesis of trichothecenes has been identified as trichodiene synthase encoded by the gene *Tri5 (15)*. Other genes playing a role in the biosynthesis of various trichothecenes have been identified as *Tri3*, *Tri4* and *Tri6 (29, 39, 57)*. Genes controlling fumonisin synthesis have been recently identified *(17)*.

HUMAN AND ANIMAL INTOXICATIONS BY *FUSARIUM* MYCOTOXINS

Ten thousands of people died of alimentary toxic aleukia (ATA) in Russia at the end of World War II. This was most probably caused by T-2 toxin intoxication *(33)*. This mycotoxin (trichothecene type A) was produced by *Fusarium sporotrichioides* when the wheat crop overwintered in the field. Human casualties after ingestion of this mycotoxin are still reported *(82)*.

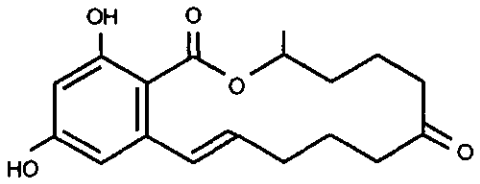


Figure 2. *Molecular structure of zearalenone.*

Substantial loss of farm animal produce is attributed to deoxynivalenol (trichothecene type B, Fig. 1). This mycotoxin, also known as vomitoxin, causes vomiting in pigs after ingestion *(64)*. Feed refusal, reduced growth of pigs and changes in milk composition of cattle are the most extensively described effects of this mycotoxin in farm animals *(5, 12, 64)*. In addition, toxic effects of the mycotoxin are observed on the immune system of animals *(5, 55, 64)*. Farm animals might become more susceptible to infectious diseases which might increase human exposure to zoonotic agents. Human intoxications following ingestion of deoxynivalenol report on vomiting and nausea *(6)*.

Zearalenone is an oestrogen (Fig. 2). It can cause infertility and pre-puberty in both male and female pigs *(14)*. Pre-puberty was observed in children in Puerto Rico, possibly caused by zearalenone ingestion *(65)*.

Fumonisin B_1 (Fig. 3) is currently the only known *Fusarium* mycotoxin with

carcinogenic properties *(23)*. This mycotoxin might play a role in the aetiology of human oesophageal cancer *(61)*. Pigs developed lung oedema (PPE) after fumonisin B_1 ingestion while this mycotoxin caused the 'hole in the head syndrome' (leucoencephalomalacia) in equine species *(19, 63)*. Basal cell hyperplasia was observed in the oesophagus of rats following exposure to *F. moniliforme* culture material with unknown levels of fumonisin B_1 contamination *(31, 36)*. Tumour promoting activity of fumonisin B_1 was observed in a rat liver bioassay *(23, 24)*. Toxic effects of fumonisin B_1 on the immune system were observed in rats *(7)*, mice *(38)*, chickens *(58)* and calves *(50)*. This may result in increased susceptibility to infectious diseases *(58)*. The mode-of-action of fumonisin B_1 has been postulated to be through the inhibition of the enzyme ceramide synthase that catalyses the acylation of sphinganine to form dihydroceramide and thus inhibits *de novo* biosynthesis of complex sphingolipids *(70)*. The relative concentration of sphinganine will increase while the relative concentration of sphingosine, one of the end-products of the sphingolipid biosynthesis, will decrease following the inhibition. The ratio of sphinganine-to-sphingosine concentrations can be determined in serum, tissues of liver and kidney and in urine and might be indicative for exposure or toxic effects *(62)*.

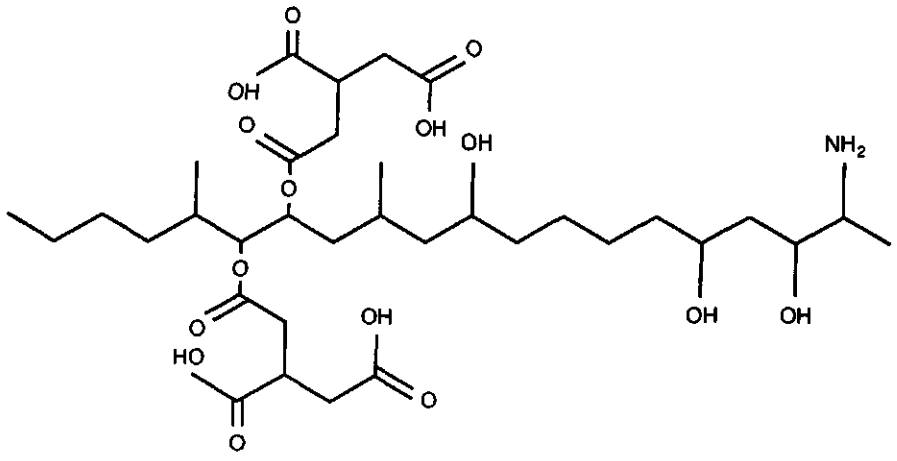


***Figure 3**. Molecular structure of fumonisin B_1.*

STABILITY OF *FUSARIUM* MYCOTOXINS AND DECONTAMINATION

The *Fusarium* mycotoxins are relatively stable under most food processing conditions and can be detected in foods *(4, 52)*. Some reduction in mycotoxin content after processing is reported but this might be due to loss of detectability rather than to degradation *(11)*. Cooking of spaghetti and noodles prepared from wheat reduced the level of deoxynivalenol to 53 % *(49)*. Addition of sodium bisulfite and L-cysteine (reducing agents) and ammonium phosphate before baking were moderately effective in reducing deoxynivalenol contamination in bread (with 38 to 46 %) *(9)*. Addition of potassium bromate or L-ascorbic acid (oxidising agents) had no effect on deoxynivalenol levels. Young *et al.* (1984) found a reduction of 35 % when baking non-yeast wheat products. T-2 toxin, zearalenone and deoxynivalenol levels of wheat, maize and soybeans, respectively, were reduced with 16, 25 and 33 % after γ-irradiation *(30)*.

Several *Fusarium* mycotoxins (zearalenone, deoxynivalenol, fumonisin B_1) can be solubilised in beer during brewing *(68)*. No mycotoxins have been detected in ethanol produced from mycotoxin contaminated cereals *(3, 8)*.

Physical cleaning of cereals by sieving out the broken and damaged kernels can reduce the mycotoxin content considerably *(72, 73)*. Since deoxynivalenol and zearalenone are not distributed uniformly in the wheat kernel, dehulling, milling out the bran and wet milling might reduce contamination levels in the resulting starch fractions considerably *(4, 73, 74)*. However, the highly contaminated bran fractions are often designated for feed production *(4)*.

Specific processes used in food preparation, such as nixtamalisation of maize dough (cooking with $Ca(OH)_2$) may lower the fumonisin B_1 concentration *(43, 71)*. Fumonisin B_1 is hydrolysed during the process but the toxicity of the hydrolysed products was comparable to the toxicity of the non treated material *(43, 76)*.

The fumonisin B_1 level was reduced by 79 % when contaminated maize was treated with ammonia. None of the materials (treated and non-treated) was positive in the *Salmonella*/microsomal mutagenicity assay *(51)*. Norred *et al.* (1991) found no reduction in toxicity when ammoniated fumonisin B_1 containing maize was fed to rats although the fumonisin B_1 contamination level was reduced by 45 % in the treated

material.

The health of farm animals will benefit from a decreased exposure to *Fusarium* mycotoxins. It will be beneficial to the immune system and might, thus, limit human exposure to zoonotic diseases. Exposure of animals to mycotoxins might be limited by adding non-nutritive adsorbent compounds to the feed *(53, 59, 60, 77)*. *In vitro* experiments showed that cholestyramine was more effective in zearalenone adsorption than crospovidone, montmorillonite, bentonite, sepiolite and magnesium trisilicate *(60)*. However, sweeteners (raw sugar, dehydrated molasses) nor bentonite could prevent toxic effects of zearalenone or nivalenol from feed *(77)*. Amending feed with hydrated sodium calcium aluminosilicate did not influence the toxic effects of the deoxynivalenol contamination *(53)*.

RISK ASSESSMENT

In the production of safe food, risk assessment is increasingly used to estimate the probability and severity of adverse health effects to the consumer following consumption of food containing a certain hazardous agent. Risk assessment is a four step procedure comprising hazard identification, exposure assessment, hazard characterisation (including dose-response assessment) and risk characterisation *(13, 21)*. The statement of purpose is described prior to the risk assessment and the risk management succeeds the risk assessment. Risk assessment should provide quantitative information on factors that may result in a certain unwanted situation. Such information is essential in managing an unacceptable situation. The protocol of risk assessment is described in Fig. 4.

Risk assessment is based on currently published scientific knowledge and the procedure needs to be repeated when new scientific research data become available. Only a clear and transparent way of identification of hazards will enable well-considered decisions to manage non-acceptable health risks.

Hazard identification is the identification of an agent of concern and evaluation of that agent as a potential hazard with food. This agent could be of microbiological, physical or chemical origin *(13, 21)*. This first step in the risk assessment of *Fusarium* mycotoxins identifies the secondary metabolites with the potential to cause

harm to the health of humans when present in food.

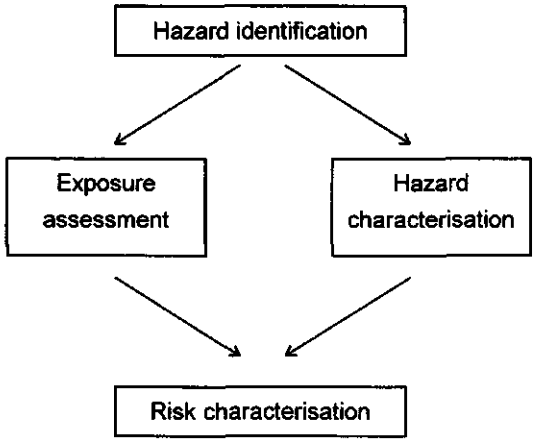


Figure 4. *Risk assessment.*

Exposure assessment is the qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposure from other sources if relevant *(13, 21)*. The exposure of humans to *Fusarium* mycotoxins is assessed by determining the levels of mycotoxin contamination in food and estimating intake of the specified foods.

Hazard characterisation is the qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical and physical agents which may be present in food *(13, 21)*. For biological agents such as *Fusarium* mycotoxins, a dose-response assessment should be performed combining data on exposure with data on toxicity.

Risk characterisation is the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known potential adverse health effects in a given population based on hazard identification, exposure assessment and hazard characterisation *(13, 21)*. The results of the before described steps in the risk assessment of *Fusarium* mycotoxins will be evaluated in this risk characterisation, with regard to the public health.

STATEMENT OF PURPOSE

The purpose of this study is to estimate the toxic effects of *Fusarium* mycotoxins present in food on the health of the population in The Netherlands by using a formal risk assessment approach.

OUTLINE OF THIS THESIS

Laboratory experiments, literature studies and modelling studies, as described in *Chapters 2* to *9*, have been conducted to support this risk assessment on *Fusarium* mycotoxins. This risk assessment starts with a general consideration of *Fusarium* species and their mycotoxins. The succeeding steps will emphasise on fumonisin B_1. This *Fusarium* mycotoxin may play a role in the aetiology of human oesophageal cancer and is, therefore, currently regarded as most significant *Fusarium* mycotoxin.

In *Chapter 2,* the hazard identification, those *Fusarium* secondary metabolites are identified that can occur in food and that have been related to human intoxications or are suspected to be toxic to humans based on toxicity in animal experiments.

The results of the exposure assessment are described in *Chapters 3* to *7*. *Fusarium* contamination of cereals grown in The Netherlands is studied *(Chapter 3)* along with the genotypic and phenotypic variability within the isolated *F. culmorum* and *F. avenaceum* strains *(Chapter 4)* since mycotoxin production is both species and strain dependant. Deoxynivalenol and zearalenone contamination are determined in cereal samples harvested in 1993 in The Netherlands *(Chapter 3)* while fumonisin B_1 contamination is determined in imported maize intended for food production and maize containing foods *(Chapters 5* and *6)*. The probability of being daily exposed to a mean quantity of fumonisin B_1 when consuming a certain amount of maize is estimated in *Chapter 7*. Groups in population at risk for high fumonisin B_1 intake are identified in this chapter.

The hazard characterisation of fumonisin B_1 is described in *Chapter 8*. A dose-response assessment is carried out on the toxic effects of oral administration of fumonisin B_1 to rats in a 28-day toxicity study. A tolerable daily intake will be estimated *(Chapter 9)* for fumonisin B_1, derived from the data from the study on

fumonisin B_1 toxicity in rats.

Finally, the results of the preceding studies are evaluated in the general discussion in *Chapter 9*, the risk assessment. Public health aspects of fumonisin B_1 occurring in food in The Netherlands are discussed in more detail.

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2

Identification of hazardous *Fusarium* secondary metabolites occurring in food raw materials[1]

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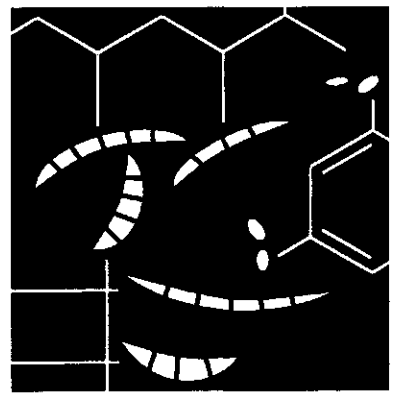

ABSTRACT

In the hazard identification, the first step of the risk assessment, hazardous secondary metabolites, mycotoxins, produced by *Fusarium* moulds, were identified. A literature survey revealed the occurrence of 61 *Fusarium* species in agricultural produce (cereals, vegetables, fruits) of which 35 species were reported to produce a total of 137 secondary metabolites in laboratory experiments. The literature review showed that 41 secondary metabolites (30 %) were tested and found toxic in various toxicity assays and should be considered hazardous if present in food. Six of these were reported to be related to human intoxications (group A). Four of the six secondary metabolites of group A, belonged to the trichothecenes (T-2 toxin, nivalenol, deoxynivalenol and acetyldeoxynivalenol), the others were zearalenone and fumonisin B_1. Twelve secondary metabolites, including the previously mentioned six, were reported to have been tested in animal feeding trials in vertebrates. All of them showed negative health effects in the test animals. These twelve secondary metabolites should be regarded as hazardous and need to primarily be evaluated in a risk assessment for *Fusarium* mycotoxins in food commodities.

[1] This chapter is a summarised version of the manuscript in *J. Food Saf. (1997) 17,161-192.* References to the tables can be found in the published manuscript

INTRODUCTION

In the production of safe food, risk assessment is increasingly used to estimate the probability that harm to the health of the consumer may occur by consumption of food containing a certain hazardous agent. Risk assessment provides quantitative information on a certain unwanted situation. Such information is essential in managing the unacceptable situation. Risk assessment is a four step procedure comprising hazard identification, exposure assessment, hazard characterisation (including dose-response assessment) and risk characterisation. The hazard identification is described in this paper, which can be defined as the identification of an agent of concern and evaluation of that agent as a potential hazard with food. This agent could be of microbiological, physical or chemical origin *(9, 10)*.

Risk assessment is based on currently (published) scientific knowledge. The procedure should be repeated when new scientific research data become available. Only a proper and clear way of identification of the hazards will enable well-considered decisions to manage the public health problems.

Currently, mycotoxins receive much attention because of their potential adverse effects to the health of humans and animals. Mycotoxins can be defined as those secondary metabolites produced by fungi which may cause harm to the consumer's health. When estimating harm caused by mycotoxins, risk assessment studies are often directed to a single mycotoxin *(5, 6, 20, 21, 42)*. Those studies generally start with exposure assessment, overlooking relevant aspects of the complex issue. This especially applies to the last step of the procedure, the risk characterisation. This step requires the consideration of all the potentially produced mycotoxins, to evaluate for preventive measures.

The first step of this risk assessment, the hazard identification, describes the mycotoxins produced by species of the fungal genus *Fusarium*. Relevant scientific literature was reviewed to identify the mycotoxins produced by *Fusarium* which may be present in food and can cause harm to human health. The fungal genus *Fusarium* comprises various species which are ubiquitous in the environment and can easily invade plant tissue, especially of agricultural produce. Production of secondary metabolites of these species is mainly limited to the field period of the

crop *(26)*. Many of these secondary metabolites have known toxic effects on human and animal health, which can vary from acute food poisoning to oesophageal or liver cancer as a result of chronic exposure *(12, 36, 43)*.

OUTLINE OF THE PROCEDURE

The here presented hazard identification of mycotoxins produced by the fungal genus *Fusarium* was carried out based on the FAO/WHO guidelines *(9, 10)*. The procedure of the hazard identification is presented in Fig. 1.

The procedure started with considering all the currently known *Fusarium* species, since the contamination of food crops with *Fusarium* mycotoxins is directly related to the occurrence of the *Fusarium* species.

In the next step in the procedure only those *Fusarium* species were considered that have been reported to occur in or on relevant food crops, cereals, fruits and vegetables. This step was followed by reviewing the literature for the production of secondary metabolites by these species in laboratory experiments. It was assumed that *Fusarium* species that can produce secondary metabolites in laboratory experiments also have the potential to produce the same group of secondary metabolites in the field, unless other information is available.

For the evaluation of the secondary metabolites as hazardous agents, data were obtained from reported disease outbreaks and from published results of toxicity tests. Several mycotoxins have been associated with human intoxications. They were listed as group A. The mycotoxins tested in toxicity experiments were listed in group B. Studies in which the mycotoxins were tested in animal experiments (vertebrates), mycotoxins belonging to group B-I, revealed most information on overall toxicity. Assays in which the toxic effects of the mycotoxins were studied in the invertebrate *Artemia salina* (brine shrimp), mycotoxins belonging to group B-II, revealed information on toxicity in one organism. The last group, B-III, contained mycotoxins tested in cell culture assays. These assays reveal information on the mechanism of toxicity or the sensitivity between cells derived from various organs or animal species.

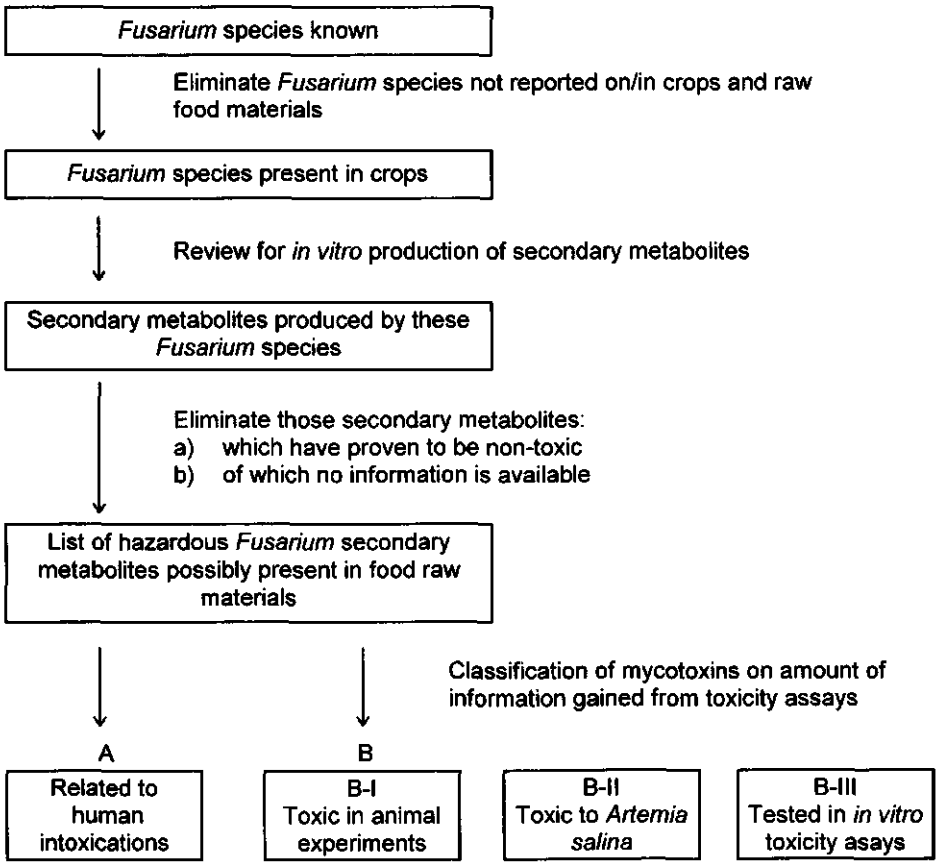

Figure 1. *Identification of* Fusarium *mycotoxins.*

RESULTS

The *Fusarium* species that have been reported to occur in food crops are relevant when mycotoxins in food are studied. According to a recent literature review, 61 *Fusarium* species have been isolated from food raw materials on a world-wide basis *(26)*. Co-occurrence of species in one crop was reported and no *Fusarium* species were restricted to a certain crop species.

Published information revealed that 35 out of these 61 *Fusarium* species could produce a total of 137 secondary metabolites under laboratory conditions *(26)*. The

results are not shown in this summarised version. Two major groups, trichothecenes (79 secondary metabolites) and other secondary metabolites (58 secondary metabolites), were distinguished. The trichothecenes were subdivided in type A (60 secondary metabolites) and type B (11 secondary metabolites), the latter characterised by a keton group on C_8, and the group of other trichothecenes (8 secondary metabolites). All these secondary metabolites should be considered as potentially present in food crops.

Table 1. *Secondary metabolites produced by* Fusarium *related to human intoxications (Group A).*

Trichothecenes type A	T-2 toxin
Trichothecenes type B	Nivalenol Deoxynivalenol Acetyldeoxynivalenol
Other mycotoxins	Zearalenone Fumonisin B_1

The *Fusarium* mycotoxins related to human intoxications are listed as group A mycotoxins in Fig. 1 and are presented in Table 1. The trichothecenes, T-2 toxin, nivalenol, deoxynivalenol (DON) and acetyldeoxynivalenol, have been related to food poisoning symptoms and human casualties *(4, 43)*. Zearalenone (ZEA) has been reported in relation to oestrogenic effects in children *(33)*, while epidemiological studies in South Africa and China revealed fumonisin B_1 (FB_1) related to the aetiology of human oesophageal cancer *(8, 13, 36)*.

A total of 41 secondary metabolites was tested (literature data) and found toxic in toxicity experiments. These secondary metabolites are referred to as mycotoxins and are presented in group B (Fig. 1).

Fusarium mycotoxins tested in vertebrates, via animal experiments, belong to group B-I (Fig. 1) and are listed in Table 2. The toxins were mainly administered by

regarded as non-producers in this study. The other 35 *Fusarium* species can produce at least 137 secondary metabolites of which 58 % belong to the group of trichothecenes.

Six *Fusarium* mycotoxins were found to be related to human intoxications (group A mycotoxins) and should, therefore, be considered principally in the risk assessment. The effects on human health can vary from acute food poisoning with human casualties to playing a role in the aetiology of oesophageal cancer as a result of chronic exposure *(17, 43)*. Extended reviews have been published on the toxic effects of several of these *Fusarium* mycotoxins. Kuiper-Goodman and co-workers (1987) reported on toxicity of ZEA. Toxicity of DON has been reviewed by Rotter *et al.* (1996) while Jackson *et al.* (1996) published on toxicity of fumonisins.

The *Fusarium* species producing the mycotoxins belonging to group A can be isolated world-wide from food raw materials. Several mycotoxins of group A can be produced simultaneously by these species, along with various other secondary metabolites *(26)*. These accompanying secondary metabolites may contribute substantially to the total toxicity expressed but have never been directly related to human intoxications *(11, 18, 19)*. Information about accompanying toxins is usually not available for various reasons. At the time of intoxications the identity of other toxins may not be known, analytical methodology and calibrants for the identification and quantification may not be available or the concentration of other toxins may be below limit of determination.

A total of 41 mycotoxins were tested in toxicity experiments and were listed as group B mycotoxins. The twelve *Fusarium* mycotoxins belonging to group B-I have been related to intoxications in farm animals and toxic effects in animal experiments (Table 2). Six of those twelve also belong to group A and, therefore, the supplementary six mycotoxins of group B-I should similarly be regarded as potentially harmful to human health. Analogously to the mycotoxins belonging to group A, the follow-up steps in the risk assessment should be considered for the supplementary six *Fusarium* mycotoxins belonging to group B-I to estimate the risks for the human population.

Animal experiments reveal information on the overall toxicity of a mycotoxin and

can point towards certain target organs. Toxicity of the mycotoxins may be tested in several cell lines derived from the target organs, both of human and animal origin, and results should be compared to determine relative sensitivity between species *(16, 22, 23)*. Cell lines have proven to be effective in research on mechanisms of toxicity *(1, 7, 31)*.

The currently most frequently used method for toxicity measurements is to test various mycotoxins in one type of assay, e.g. in an invertebrate (*Artemia salina*) or cell line *(16, 38)*. This approach has some important drawbacks. The solubility of the mycotoxins is a major constraint to the use of these assays. Cell lines and *Artemia salina* may be sensitive to organic solvents at certain concentrations. Hydrophobic mycotoxins can, therefore, not be studied in these assays. The pH of the medium may also affect the toxicity *(14)*. Therefore, when comparing toxicity of various mycotoxins using cell lines or invertebrates, these mycotoxins should, preferably, have identical chemical characteristics.

In summary, the procedure of hazard identification of secondary metabolites produced by species of the fungal genus *Fusarium* revealed a total of 137 secondary metabolites, possibly present in food raw materials. A total of 41 of those secondary metabolites was toxic in various toxicity assays. The study on toxicity revealed six secondary metabolites, group A, that were related to human intoxications. These six secondary metabolites should be regarded as most important of the group of *Fusarium* mycotoxins. A total of twelve secondary metabolites (including those belonging to group A) was found of which the toxicity was characterised in animal (vertebrate) studies. These mycotoxins can all be produced by *Fusarium* species that have been isolated from food raw materials and should, therefore, be considered as potentially present in food and thus posing a threat to human health.

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3

Fungal infection and presence of deoxynivalenol and zearalenone in cereals grown in The Netherlands

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ABSTRACT

In 1991 and 1993 cereals were sampled during harvest in The Netherlands. The samples were tested for the presence of moulds and the samples of 1993 were additionally tested for contamination with the mycotoxins deoxynivalenol and zearalenone. The moulds were identified to genus level and those belonging to the genus *Fusarium* to species level. The total fungal infection of cereals in 1991 did not differ from 1993, with a median value of 5.0 log CFU g^{-1} in both years. The incidences of the genera *Aspergillus*, *Penicillium*, the group of *Mucor* and *Rhizopus*, *Cladosporium,* and *Fusarium* differed considerably between the two years, possibly caused by the different weather conditions. The numbers of samples infected with *Fusarium* were much higher in 1993 (83 %) than in 1991 (34 %). In 1991, no *Fusarium* was detected in samples from the southern part of The Netherlands, as opposed to 1993, when *Fusarium* was found in all regions sampled. The most dominant *Fusarium* species in 1991 were *Fusarium culmorum* and *Fusarium avenaceum.* In 1993, *Fusarium poae*, *Fusarium culmorum* and *Fusarium crookwellense* dominated. All these *Fusarium* species are known mycotoxin producers. Three percent of the cereal samples of 1993 contained deoxynivalenol

and 1 % contained zearalenone in levels of over 500 ng g^{-1} and 200 ng g^{-1}, respectively. This study has shown that the incidences of various fungal genera and *Fusarium* species in cereals in The Netherlands can vary from year to year. Considerable numbers of toxigenic *Fusarium* moulds can occur and *Fusarium* mycotoxins may be present at harvest.

INTRODUCTION

The negative effects of fungal infection in cereal crops have traditionally been related to loss of grain yields and decrease of processing quality *(2, 5)*. Attention is currently focused on food safety due to the presence of toxigenic fungi in the crops. The toxic metabolites excreted by these fungi can adversely influence the health of the consumers of those cereals *(18, 27)*.

Fusarium is generally regarded as the most important invasive fungus in cereals in the temperate climate zone *(10, 21)*. Species of this genus are able to excrete toxic secondary metabolites in the plant during the field period of grain crops. The most often occurring *Fusarium* mycotoxins are deoxynivalenol (DON), causing growth depletion in pigs and immunosuppression in laboratory animals, and zearalenone (ZEA), causing hyperoestrogenism in female pigs and possible precocious pubertal changes in children *(15)*. Changing agricultural practices can affect the incidence of the infection and the genera present in cereal crops over the years *(12)*. The increase in the use of fertilisers and the acquired resistance of moulds against fungicides are presumably the major causes for the increase of *Fusarium* infection in wheat *(5)*. Dry storage conditions after harvest limit the growth of the *Fusarium* mycelium, but mycotoxins may be produced by the mycelium present in and on the grain kernels at the moment of harvest *(10)*. Most of the mycotoxins in the cereals are stable under storage and process conditions and may enter final food and feed products *(17, 20)*. Due to the amount and seasonal distribution of rainfall, *Fusarium* infection of cereals can fluctuate from year to year and even during the growth season *(13, 24)*. High rainfall creates favourable conditions for *Fusarium* growth and spreading within the crop by splash dispersal of conidia *(8)*. Other factors influencing *Fusarium* infection and diversity of *Fusarium*

species are temperature *(6)*, crop rotation *(4)*, insect epidemics *(8)*, quality of seed *(6)*, fungicides applied *(12)*, susceptibility of plant cultivar used *(1)* and geographic region *(6, 26)*.

This study presents the results of surveys carried out in 1991 and 1993 on the fungal infection of cereals in The Netherlands sampled at time of harvest, July through September. Total fungal infection and shift in occurring genera were determined as was the influence of climate and geographic regions on the occurrence of *Fusarium*. *Fusarium* isolates were morphologically identified to species level. The cereal samples of 1993 were examined for the mycotoxins DON and ZEA. The aim was to study the presence and abundance of toxinogenic *Fusarium* species and the presence of two mycotoxins in cereals grown in The Netherlands to obtain an impression on the mycological and toxicological quality of raw cereal materials intended for food and feed production. The results of the mycotoxin analysis will be compared with other studies carried out with cereals grown in The Netherlands from crop years 1984/1985 *(23)* and 1988/1989 *(25)*.

MATERIALS AND METHODS

Sample collection

Grain samples, wheat, barley, oats, rye and triticale, of approximately 1 kg were collected *at random* in The Netherlands in 1991 and 1993. The samples were taken at the moment of harvest, July through September, and were transported to the laboratory within 1 day. The cereal samples were stored at room temperature and analysed within one week in 1991. Those taken in 1993 were analysed within one day.

Colony-forming units (CFU)

A subsample of 25 g was crushed for 1 min at high speed in a blender beaker with 225 ml of sterile 0.9 % NaCl solution. Tenfold serial dilutions were made in 1 % peptone solution. An aliquot of 0.1 ml of the dilutions was surface plated, in triplicate, on oxytetracycline glucose yeast extract agar composed of 2 % (w/v) glucose (Difco Laboratories, Detroit, MI, USA), 0.5 % (w/v) yeast extract (Difco), and 0.1 mg of

terramycin ml^{-1} (Pfizer, New York, NY, USA). The plates (15 cm diam.) were incubated in an upright position at 25°C for 5 days in the dark. The total number of CFU were counted on dilution plates with between 10 and 100 colonies. It has to be taken into account that colonies can arise from a single spore or a clump of spores or from fragments of mycelium. Fungi were counted individually. Fungi of the genera *Fusarium*, *Cladosporium*, *Aspergillus*, *Penicillium,* and the group of *Mucor* and *Rhizopus* were identified by microscopy. The frequency distribution (%) of these genera was expressed as the number of samples infected with one or more colonies of a genus as a percentage of the total number of cereal samples. *Fusarium* moulds were isolated by subculturing suspected colonies on 2 % (w/v) malt extract (Oxoid, Bansingstoke, UK) agar for 5 days. The *Fusarium* isolates were identified after plating on Synthetischer nährstoffarmer Agar, composed of a 0.02 % (w/v) saccharose and glucose, 0.05 % (w/v) KCl and $MgSO_4.7H_2O$, and 0.1 % (w/v) KNO_3 and KH_2PO_4 and on 3 % (w/v) oatmeal agar according to Nelson *et al.* (1983) and were confirmed by Centraalbureau voor Schimmelcultures (Baarn, The Netherlands).

Deoxynivalenol and zearalenone assays

Cereals were ground in a Retsch mill equipped with a 0.5-mm aperture sieve. Flour aliquots of 50 g and 5 g were examined for DON and ZEA, respectively, using ELISA (enzyme-linked immunosorbent assays) (Veratox, NEOGEN corp., Lansing, MI, USA). The assays were carried out according to the manufacturer's instructions. Some minor adjustments were implemented. Extraction of DON was carried out in an Erlenmeyer flask containing 50 g of sample and 250 ml of distilled water, placed on a reciprocal shaker (130 rpm) for 30 min. In both assays, the optical density was measured at 660 nm, which was the standard filter of the microtiterplate reader, instead of 650 nm. An additional standard solution, equivalent to 200 ng ZEA g^{-1}, was provided by the manufacturer. The standards with the lowest amounts of mycotoxins, equivalent to 500 ng DON g^{-1} and 200 ng ZEA g^{-1}, as provided by the manufacturer, were regarded as the lower limit of determination. The incidences of samples containing the mycotoxins DON or ZEA were, therefore, expressed as

percentage of samples contaminated with levels over 500 ng DON g^{-1} or over 200 ng ZEA g^{-1}, respectively.

Climatic conditions

Data on temperature and rainfall in the growing seasons were obtained from the Royal Netherlands Meteorological Institute (KNMI) *(9)*. Average monthly temperatures and rainfall were calculated according to the method recommended by KNMI using the data obtained at weather stations situated in the five geographic regions in The Netherlands where the cereals were sampled. The data were compared to average data measured in the period 1961 to 1990 at the same weather stations. The ratio of the number of months with higher temperature than average to the number of months with lower temperature than average was determined and expressed as R_T. Similar calculations were carried out for the rainfall and expressed as R_r. R_T and R_r were calculated for the growing season (preceding year July until September in the year of harvest) as well as for the period before and during harvest (April till September) in both sampling years.

RESULTS

The fungal infection of cereals in The Netherlands in 1991 and 1993 at harvest is presented in Fig. 1. In this figure, the frequency distribution of CFU is presented as log CFU g^{-1} fresh weight. In both sampling years, about 40 to 50 % of the samples contained 4 to 5 log CFU g^{-1} and 40 to 50 % of the samples contained 5 to 6 log CFU g^{-1}. The infection varied from 3.3 to 7.3 log CFU g^{-1} in 1991 and from 4.1 to 6.3 log CFU g^{-1} in 1993, with a median value of 5.0 log CFU g^{-1} in both years.

The frequency distribution of the fungal genera, defined as the ratio of the number of samples infected by the genus to the total number of samples *(22)*, is shown in Table 1. The infection with *Cladosporium* and *Fusarium* was higher in 1993 than in 1991. The occurrence of the genera *Aspergillus*, *Penicillium*, and the group of *Mucor* and *Rhizopus* was higher in 1991.

The distribution of *Fusarium* infection in 1991 and 1993 in The Netherlands is presented in Fig. 2. In all five regions, *Fusarium* infection was much higher in 1993

than in 1991. The most distinct results were found in region E where no *Fusarium* infection was detected in 1991 as opposed to 44 % positive samples in 1993.

In 1991, 45 *Fusarium* isolates were collected and these isolates belonged to six species. In 1993, 290 *Fusarium* isolates were obtained, of which 56 were morphologically identified. The frequency distribution of the *Fusarium* species is shown in Table 2. As shown, overall, eight different *Fusarium* species were found. The relative occurrence of the different species varied considerably between the two sampling years. In Table 3, the potential of these species to produce mycotoxins is presented.

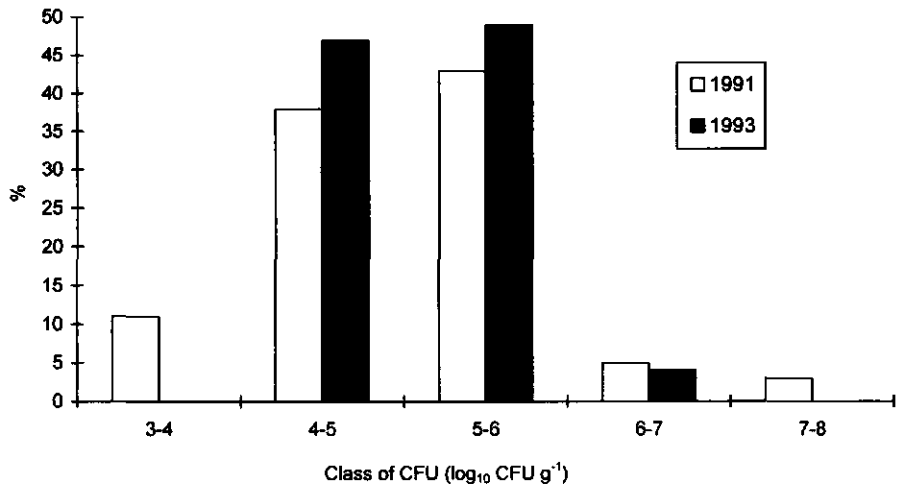

Figure 1. *Frequency distribution of fungal infection of cereals sampled in The Netherlands at harvest in 1991 and 1993.*

DON levels of 500 ng g^{-1} and up, were detected in two wheat samples, 3 % of the total cereal samples obtained in 1993. The levels found were 500 and 750 ng DON g^{-1} of cereal. ZEA was present in levels over 200 ng g^{-1} in one barley sample (270 ng ZEA g^{-1}), 1 % of the samples tested. The two wheat samples were infected with 5.4 and 5.5 log CFU g^{-1}. The barley sample, positive for ZEA, had a count of 5.7 log CFU g^{-1}. One wheat sample and the barley sample originated from region D, the other wheat sample from region A.

Table 1. *Frequency distribution of different fungal genera isolated from cereals in The Netherlands in 1991 and 1993.*

Genus	Contaminated samples (%)	
	1991 (n=65)[a]	1993 (n=69)
Aspergillus	29	3
Penicillium	65	20
Mucor/Rhizopus	35	30
Cladosporium	89	100
Fusarium	34	83
Other fungi	92	97

[a] n is total number of cereal samples

Table 2. *Frequency distribution of* Fusarium *species isolated from cereals in The Netherlands.*

Fusarium section	*Fusarium* species	Occurring species (%)	
		1991 (n=45)[a]	1993 (n=56)
Discolor	*F. crookwellense/cerealis*	0	11
	F. culmorum	40	25
	F. graminearum	2	5
	F. sambucinum	2	11
Sporotrichiella	*F. poae*	13	39
	F. tricinctum	0	2
Roseum	*F. avenaceum*	38	7
Gibbosum	*F. acuminatum*	4	0

[a] n is total number of *Fusarium* isolates identified

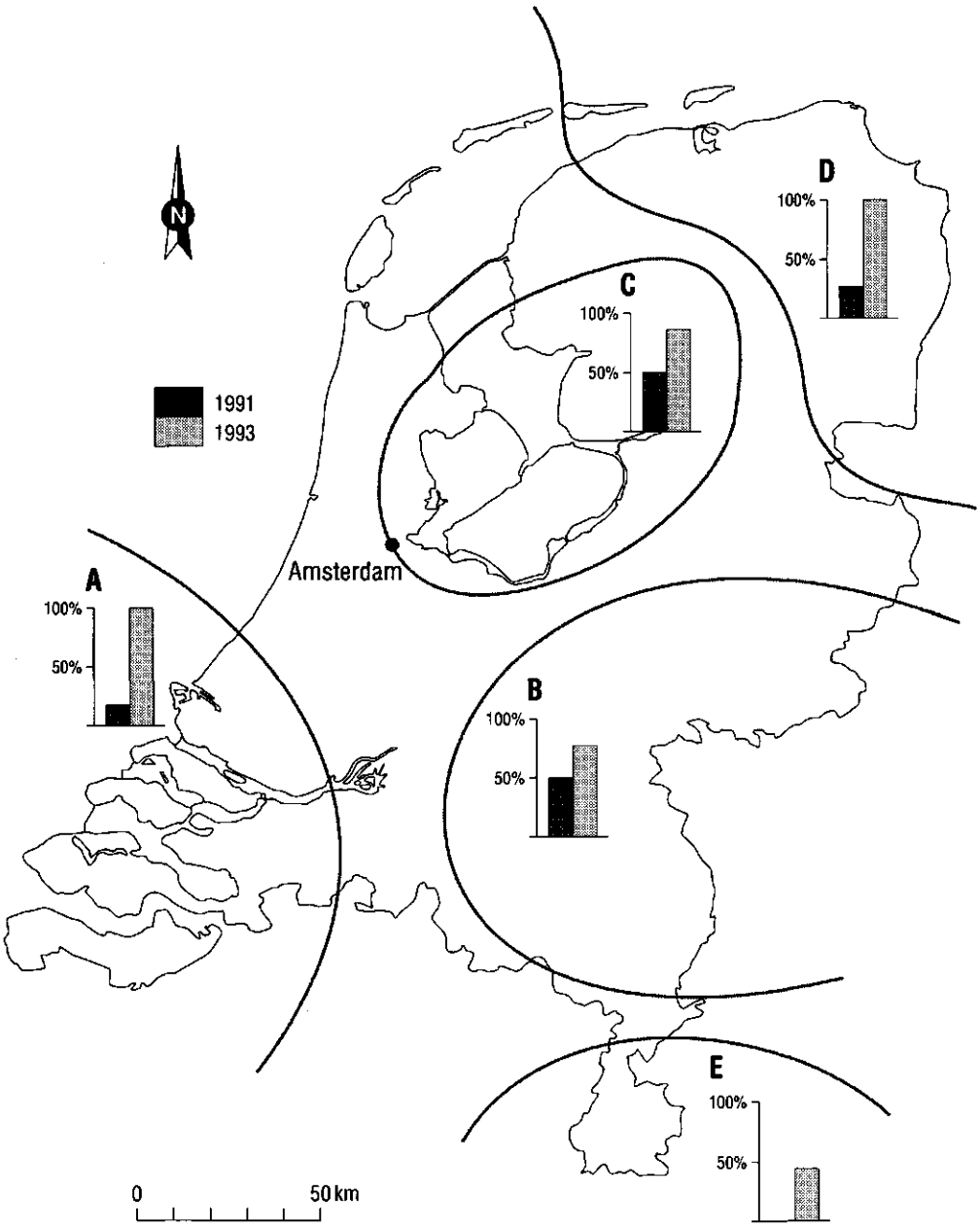

Figure 2. *Frequency distribution of* Fusarium *infection of cereals in five regions in The Netherlands in 1991 and 1993.*

Table 3. *Literature overview of mycotoxins produced by* Fusarium *species isolated from cereals in The Netherlands*[*].

Fusarium species	Mycotoxins produced
F. acuminatum	Zearalenone, neosolaniol, diacetoxyscirpenol, T-2 toxin, deoxynivalenol, chlamydosporol, moniliformin
F. avenaceum	Zearalenone, nivalenol, deoxynivalenol, fusarin C, moniliformin
F. crookwellense/cerealis	Zearalenone, trichodermin derivatives, sambucinol, diacetoxyscirpenol, T-2 toxin, nivalenol derivatives, butenolide, culmorin, fusarin C, sambucoin
F. culmorum	Zearalenone, calonectrin, sambucinol, diacetoxyscirpenol, deoxynivalenol, butenolide, culmorin, sambucoin
F. graminearum	Zearalenone, sambucinol, diacetoxyscirpenol, T-2 toxin, nivalenol, deoxynivalenol, butenolide, culmorin
F. poae	Diacetoxyscirpenol, T-2 toxin, nivalenol, fusarin C
F. sambucinum	Zearalenone, neosolaniol, diacetoxyscirpenol, T-2 toxin, nivalenol, deoxynivalenol, butenolide
F. tricinctum	Neosolaniol, diacetoxyscirpenol, T-2 tetraol, T-2 toxin

[*] de Nijs *et al.* (1997) *Chapter 2*

R_T ratios in the growing seasons were 2.8 in 1991 and 2.5 in 1993, indicating that the sampling year 1991 had almost three times more months with higher temperature during the growing season than average, while 1993 had two-and-a-half times more months with higher temperatures than average. Ratios for rainfall, R_r, in the growing seasons were 0.3 and 0.9 for 1991 and 1993, respectively. In the period April through September, R_T was 2 and R_r was 0.2 in 1991, while in 1993 both R_T and R_r were 1 during this period. This indicates a dry harvest season with high temperatures in 1991 compared to average weather conditions in 1993. Table 4 shows the average seasonal temperature and rainfall over a 30-year period (1961-1990) in The Netherlands.

containing a high level of ZEA.

Currently, there is no legislation regarding maximum levels of DON and ZEA in cereals in the European Community. A guideline of 1,000 ng DON g^{-1} of cereal is suggested, and 200 ng ZEA g^{-1} in cereals intended for food production *(11)*. None of the samples in this study contained over 1,000 ng DON g^{-1} and one sample contained ZEA above the mentioned limit. In 1990, Tanaka and co-workers *(23)* investigated 28 cereal samples grown in The Netherlands (wheat, barley, rye, oats and triticale) for the presence of DON and ZEA. Eighty-nine percent of the samples contained DON and 61 % contained ZEA. Out of 22 cereal samples, harvested in The Netherlands and examined by Veldman and co-workers *(25)*, 14 % contained DON and 9 % ZEA. The levels of contamination in these two studies were all below the limits of determination as set in this study with the exception of one wheat sample, which contained 512 ng DON g^{-1}. Together with the results of our survey, this shows that, incidentally, high levels of *Fusarium* mycotoxins can be detected in cereals grown in The Netherlands. This is reason for concern, since part of the cereals is intended for food production.

Incidences of fungal genera can vary from year to year in cereals grown in The Netherlands. The cereals may be infected with considerable numbers of toxigenic *Fusarium* spp. and significant concentrations of *Fusarium* mycotoxins. The number of *Fusarium* species present can vary from year to year and might be influenced by climatic conditions. New *Fusarium* species were possibly introduced in a previously non-infected region, possibly through seed contaminated with *Fusarium* fungal parts. The observed concentrations of *Fusarium* mycotoxins in the cereals could have a considerable impact on the safety of food and feed and subsequently on the public health. It is, therefore, of interest to monitor fungal contamination of cereals in The Netherlands for the presence of toxigenic fungi and to determine the presence of *Fusarium* mycotoxins in the cereals.

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4

Variations in random amplified polymorphic DNA patterns and secondary metabolite profiles within *Fusarium* species from cereals from various parts of The Netherlands

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ABSTRACT

Random amplified polymorphic DNA pattern (RAPD pattern) analysis and the secondary metabolite profile (SM profile) assays were used to investigate genetic variability within *Fusarium culmorum* and *Fusarium avenaceum* isolated from four geographic regions in The Netherlands. The aim was to investigate the genotypic and phenotypic variability within the two species in relation to geographic origin. The occurrence of isolates with a certain characteristic set of RAPD pattern and SM profile could have predictive values on the mycotoxins found in the cereals originating from certain geographic regions. Among the *F. culmorum* isolates, small variations were found with RAPD pattern analysis. *F. culmorum* isolates belonging to the RAPD type I were found in all four geographic regions in The Netherlands while isolates with RAPD types Ia and Ib were obtained from region B only. All *F. culmorum* isolates showed a different SM profile. *F. avenaceum* isolates were very heterogeneous, both with RAPD pattern analysis and SM profile assay, resulting in individual identification of each isolate. It was concluded that the combination of RAPD pattern analysis and SM profile assay is very powerful to differentiate *Fusarium* isolates, permitting visualisation of variations between occurring isolates.

However, due to the high discrimination power of both assays, the application for epidemiological procedures seems to be limited.

INTRODUCTION

Fungi of the plant-pathogenic genus *Fusarium* can excrete secondary metabolites in the crop during the field period. Many of these metabolites can adversely affect the health of man and animals. The number and quantities of these mycotoxins produced and the pathogenicity towards host plants, can vary markedly between but also within *Fusarium* species *(3)*. The use of morphological characteristics of *Fusarium* colonies is restricted to identification at species level *(7)*. Groups of highly infectious isolates can be distinguished within the species by using pathogenicity tests towards host plants or vegetative compatibility grouping *(1)*. However, when characterisation of individual isolates within the species is needed, as in studies on mycotoxin production or geographic distribution of certain hazardous *Fusarium* strains, other identification techniques are requested.

Methods for describing individual isolates should visualise variations in the characteristics between the isolates. Therefore, at least two methods, based on different characteristics, may be required to assure occurrence of dissimilarities in the characteristics studied. Combination of the results of the methods will increase discrimination power. This is fundamentally different from the methods used for description of species or infectious groups, which focus on detection of identical characteristics.

In the presented experiments, we applied both the random amplified polymorphic DNA pattern (RAPD pattern) analysis and the secondary metabolite profile (SM profile) assay, which are based on genotypic and phenotypic characteristics, respectively. The RAPD pattern analysis visualises variations in the total DNA and is therefore suitable for differentiation of *Fusarium* isolates below species level *(6, 11, 15)*. The SM profile assay demonstrates variations in profiles of secondary metabolites excreted by fungal cultures in solid culture media *(4, 14)*. Both the RAPD pattern analysis and the SM profile assay do not require extensive trials with plants or a large variety of different experimental devices and standards.

They can easily be standardised and libraries can be established for comparison purposes.

Fusarium culmorum and *Fusarium avenaceum* are two of the most common *Fusarium* species infecting cereals in The Netherlands *(9)*. The current knowledge on genotypic and phenotypic variations among isolates of these species obtained from cereals in The Netherlands is very limited, as well as the geographic occurrence of certain isolates. Therefore, in the present study, isolates belonging to these two species obtained in The Netherlands in the same harvest year, were tested for RAPD patterns and SM profiles and both results were combined. Furthermore, the distribution of certain strains in various geographic regions of The Netherlands was studied.

MATERIALS AND METHODS

Fusarium *isolates*

Thirty-five *Fusarium* isolates were obtained at harvest from cereal samples (wheat, barley, oats, rye, triticale) grown in four regions (A through D) in The Netherlands in 1991 as described elsewhere *(9)*. Region A indicates the south-west part, region B the central eastern part, region C the centre and region D the north-eastern part of The Netherlands. Isolation was carried out using dilution plating, starting from 25 g cereal samples on oxytetracyclin-glucose-yeast extract agar (OGYA) using Petri dishes of 15 cm diameter *(13)*. Conidia were suspended in sterile condensed milk, lyophilised and stored at room temperature. The identification numbers of *Fusarium* isolates and the cereal samples are listed in Table 1.

Morphological identification of Fusarium *isolates*

Resuscitated conidia of each *Fusarium* isolate were inoculated on two plates: oatmeal agar (OA) (2 % (w/v) oatmeal) and Spezieller nährstoffarmer agar (SNA) (0.02 % (w/v) saccharose and glucose, 0.05 % (w/v) KCl and $MgSO_4.7H_2O$ and 0.1 % (w/v) KNO_3 and KH_2PO_4) using Petri dishes of 9 cm diameter *(10)*. Each plate of SNA was supplied with three small pieces of sterile filter paper to promote sporulation. One plate of each medium was incubated at 20-25°C in daylight and

one at 25°C in darkness for at least 14 days. Morphological identification was carried out at the Centraalbureau voor Schimmelcultures in Baarn, The Netherlands.

Table 1. Fusarium *isolates used in RAPD experiments and their RAPD grouping.*

Number	*Fusarium* species	Isolate ID number	Source	Region	RAPD set
1	*Fusarium culmorum*	LWLF04-2[1]	barley	C	I
2	*F. culmorum*	LWLF04-6	barley	C	I
3	*F. culmorum*	LWLF09-2	barley	B	Ia
4	*F. culmorum*	LWLF10-2	oats	B	I
5	*F. culmorum*	LWLF16-4	wheat	B	I
6	*F. culmorum*	LWLF23-5	wheat	A	I
7	*F. culmorum*	LWLF25-6	barley	A	I
8	*F. culmorum*	LWLF29-4	wheat	A	I
9	*F. culmorum*	LWLF29-5	wheat	A	I
10	*F. culmorum*	LWLF42-1	wheat	D	I
11	*F. culmorum*	LWLF51-1	barley	B	Ib
12	*F. culmorum*	LWLF51-2	barley	B	Ib
13	*F. culmorum*	LWLF54-1	wheat	B	Ia
14	*F. culmorum*	LWLF57-1	triticale	B	I
15	*F. culmorum*	LWLF57-2	triticale	B	I
16	*F. culmorum*	LWLF64-2	barley	A	I
17	*F. culmorum*	LWLF64-3	barley	A	I
18	*F. culmorum*	LWLF64-4	barley	A	I
19	*Fusarium avenaceum*	LWLF06-4	barley	C	II
20	*F. avenaceum*	LWLF06-5	barley	C	II
21	*F. avenaceum*	LWLF16-5	wheat	B	III
22	*F. avenaceum*	LWLF38-6	wheat	B	IV
23	*F. avenaceum*	LWLF38-7	wheat	B	IV
24	*F. avenaceum*	LWLF10-4	oats	B	V
25	*F. avenaceum*	LWLF38-4	wheat	B	VI
26	*F. avenaceum*	LWLF46-3	rye	D	VI
27	*F. avenaceum*	LWLF38-3	wheat	B	VII
28	*F. avenaceum*	LWLF38-5	wheat	B	VII
29	*F. avenaceum*	LWLF08-5	triticale	B	VIII
30	*F. avenaceum*	LWLF10-3	oats	B	IX
31	*F. avenaceum*	LWLF46-2	rye	D	X
32	*F. avenaceum*	LWLF46-4	rye	D	XI
33	*F. avenaceum*	LWLF47-1	rye	D	XII
34	*F. avenaceum*	LWLF47-4	rye	D	XIII
35	*F. avenaceum*	LWLF47-5	rye	D	XIV

[1] LWLF numbers describe cereal sample and *Fusarium* isolate (e.g. LWLF04-2 is the second isolate from sample 4)

RAPD pattern analysis

DNA isolation and RAPD pattern analysis were carried as described elsewhere *(8)*. Three primers were randomly chosen and synthesised by standard phosphoramidate chemistry on a 'Gene Assembler' (Perkin Elmer Cetus, Norwalk). Decamer primers used were HLWL74 (5'-ACGTATCTGC-3'), HLWL82 (5'-CGGCCTCTGC-3') and HLWL85 (5'-ACAACTGCTC-3'). RAPD patterns were generated with these three primers for the 35 *Fusarium* isolates. The patterns were grouped into RAPD pattern sets by combining the RAPD patterns generated for all three primers. Comparison was based on prominent bands.

SM profile assay

The assay was carried out according to Frisvad and Thrane (1987) with some minor modifications. Fungal cultures were inoculated on four media in triplicate: rice meal agar (RA) (7.5 % (w/v) ricemeal (Nutana), 2 % (w/v) agar), potato-sucrose agar (PSA) (500 ml potato extract (1800 g peeled and sliced potatoes boiled in 4.5 l distilled water, 10 min, and filtered) in 1000 ml, 2 % (w/v) sucrose (BDH), 1.5 % (w/v) agar, pH 6.7), both RA and PSA amended with $1x10^{-3}$ % (w/v) $ZnSO_4.7H_2O$ and $0.5x10^{-3}$ % (w/v) $CuSO_4.5H_2O$; yeast extract-sucrose agar (YES) (2.3 % (w/v) yeast extract (Difco), 17 % (w/v) sucrose, 2.3 % (w/v) agar, pH 6.5), and Sigma yeast extract-sucrose agar (SYES) (2.3 % (w/v) yeast extract (Sigma), 17 % (w/v) sucrose (BDH), 2.3 % (w/v) agar, pH 7.0), both YES and SYES amended with 0.06 % (w/v) $MgSO_4.7H_2O$, $1.1x10^{-3}$ % (w/v) $ZnSO_4.7H_2O$ and $0.6x10^{-3}$ % (w/v) $CuSO_4.5H_2O$. The plates were incubated at 25°C for 14 days in dark. For each isolate, mycelium and the agar of all three replicates of the four agar media were placed in the same Stomacher bag. The samples were either used directly or after storage at -20°C. To this material, a volume of 75 ml chloroform-methanol (2:1 (v/v)), 75 ml ethyl acetate and one Pasteur pipette full of formic acid were added. The sample was homogenised for 4 min in a Stomacher Colworth 400. The suspension was filtered over filter paper Whatman PS no. 1 and the filtrate was vacuum-dried in a rotary evaporator at a temperature not exceeding 38°C. The residue was dissolved in two Pasteur pipette volumes of methanol (HPLC grade) and transferred to a screw cap

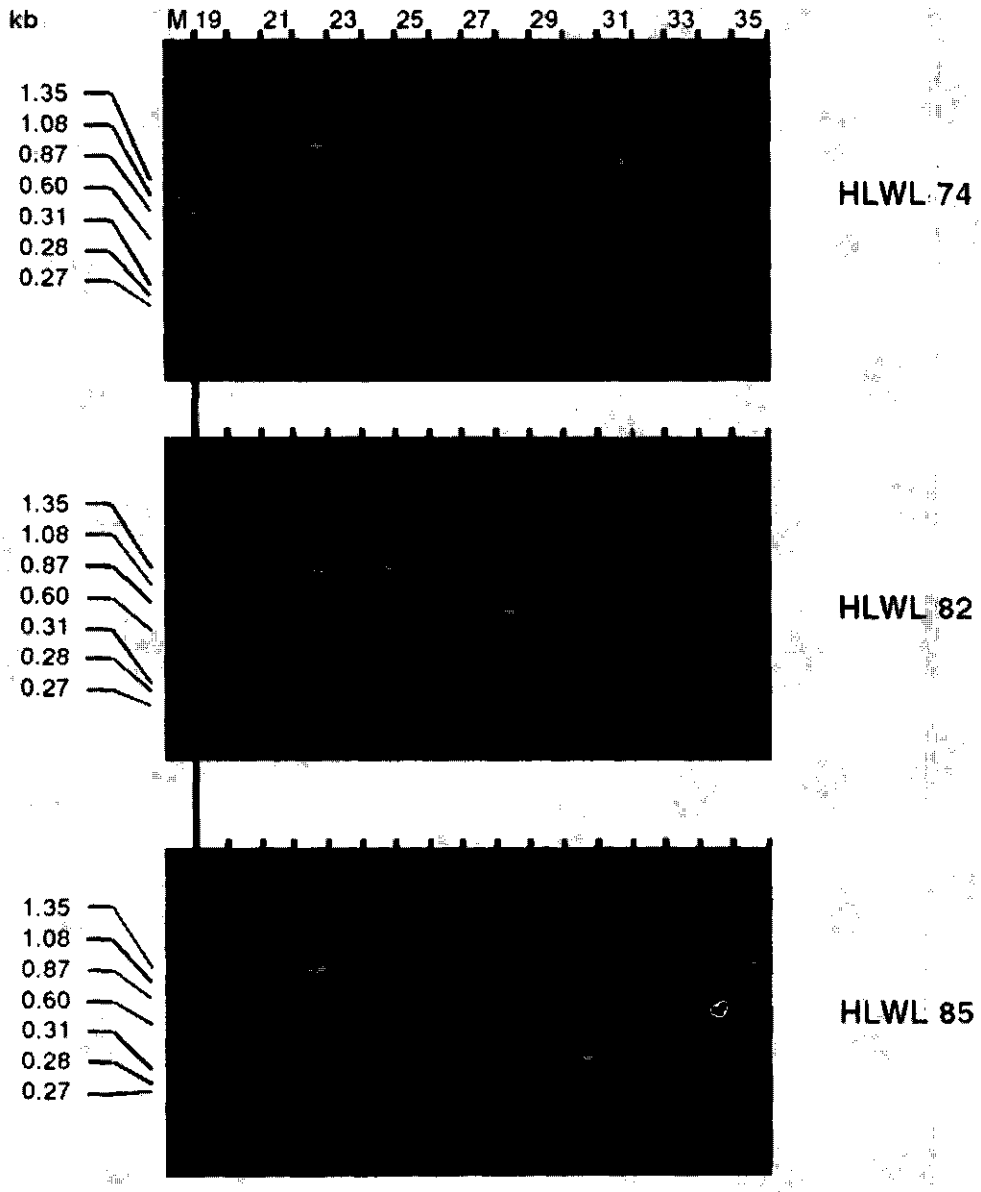

***Figure 2**. RAPD patterns of 17* Fusarium avenaceum *isolates, numbers 19-35, with primers HLWL74, HLWL82 and HLWL85. Numbers in the figure correspond with those in Table 1. Lane M contains molecular weight marker (Kb).*

Both RAPD pattern sets and SM profiles obtained for *F. avenaceum* isolates were very diverse and, when combined, allowed to recognise individual isolates within the species. All *F. avenaceum* isolates had their own, specific combination of RAPD pattern set and SM profile and, therefore, no conclusions could be drawn on the geographic distribution of certain well characterised isolates.

Various isolates of *F. avenaceum* with different RAPD pattern sets and SM profiles could be isolated from one cereal sample. It has been known that *F. avenaceum* is also morphologically variable *(7)*. A possible explanation for these results might be the presence of the teleomorph of *F. avenaceum*, *Gibberella avenacea*, in cereals in The Netherlands *(2)*. This could result in a free combination of genetic traits within the species. No teleomorph is currently known for *F. culmorum* and this may explain the lower variability within this species.

This study has shown that there is a considerable genotypic and phenotypic variability among *Fusarium* isolates belonging to two *Fusarium* species obtained from cereals originating from different geographic regions in The Netherlands. The combined use of RAPD pattern analysis and SM profile assay proved to be powerful and could be used to describe the *Fusarium* isolates individually. However, due to the high discrimination power of both assays, the application for epidemiological procedures seems to be limited. For such applications less powerful assays seems to be preferable.

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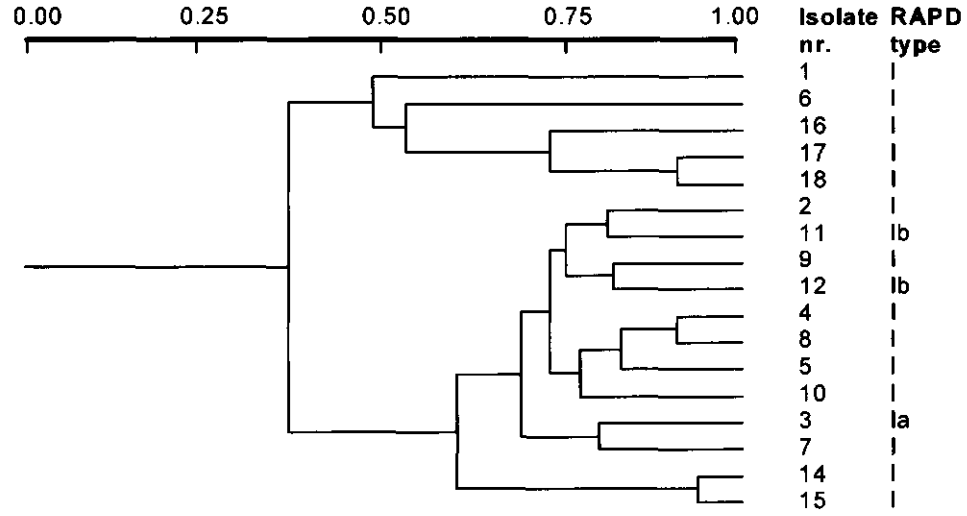

Figure 3. *Cluster analysis of SM profiles of* Fusarium culmorum, *based on Jaccard indices.*

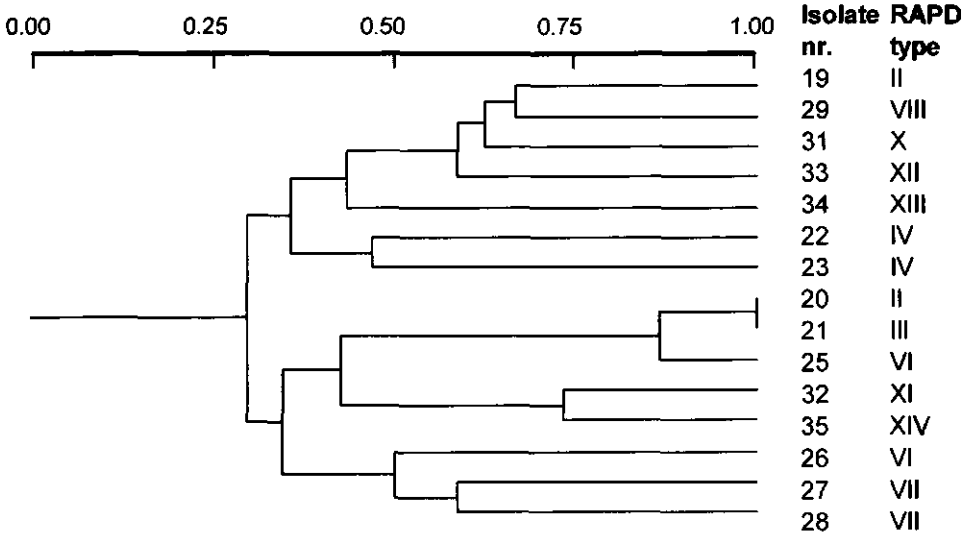

Figure 4. *Cluster analysis of SM profiles of* Fusarium avenaceum, *based on Jaccard indices.*

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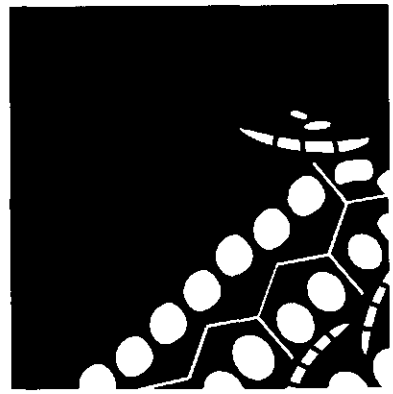
5

Fumonisin B_1 in maize for food production imported in The Netherlands



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Food Addit. Contam. (1998) in press

ABSTRACT

Sixty-two samples of maize imported in The Netherlands and intended for human consumption were analysed for fumonisin B_1 contamination. Fumonisin B_1 was detected in sixty-one of the samples in mass fractions ranging from 8 to 3,350 ng fumonisin B_1 g^{-1}. Eleven maize samples were contaminated with fumonisin B_1 in mass fractions over 1,000 ng fumonisin B_1 g^{-1}. The average contamination was 640 ng fumonisin B_1 g^{-1} maize for the positive samples and 620 ng fumonisin B_1 g^{-1} maize for all samples. Median mass fraction was 550 ng fumonisin B_1 g^{-1} maize. The results were comparable to results from other studies in maize from various countries.

INTRODUCTION

Fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium moniliforme*, was first demonstrated in maize in South Africa by Gelderblom *et al.* (1988) and has since then been found in maize grown world-wide *(11)*. FB_1 has, occasionally, been detected in rice, sorghum and navy beans *(5, 8, 12)*. The FB_1 present in the crop at harvest is not inactivated during processing conditions common in the food industry,

e.g., changes in temperature, pH and salt concentration *(6)*.

In animal experiments, FB_1 shows acute toxic effects *(2)*. In man, acute toxic effects caused by FB_1 ingestion have never been reported. The mycotoxin has carcinogenic properties in test animals and, possibly, plays a role in the aetiology of human oesophageal cancer *(4)*. Currently, a comprehensive study is carried out in the USA on the effects of chronic intake of FB_1 by rats *(7)*. Since FB_1 is most often detected in maize and is not removed or inactivated by processing, maize is regarded as most important source for human exposure to FB_1 *(9)*.

Currently, there is no legislation in the European Union on FB_1 contamination limits, but initial discussions have started. The only country with a proposed tolerance at this moment is Switzerland where a limit for the sum of fumonisins B_1 and B_2 in maize intended for human consumption has been set at 1,000 ng FB_1 g^{-1} *(3)*.

In The Netherlands all maize for food production and most for feed production is imported, since climatic conditions do not allow the growth of maize for these purposes. This paper describes the occurrence of FB_1 in maize imported in The Netherlands and intended for food production. The data contribute to the exposure assessment of the Netherlands population to FB_1. Exposure assessment is defined as the qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant *(1)*. In case of FB_1 it requires quantification of the mycotoxin in maize imported in the country. Exposure assessment is an important step in the risk assessment of humans to FB_1.

MATERIALS AND METHODS

Samples

Sixty-two maize samples (kernels) were obtained from industries in The Netherlands. The collected samples were from batches intended for food production. A part of the samples was waxy maize, containing no amylose in the starch. The samples were stored and transported at room temperature and refrigerated at approximately 4°C upon arrival in the laboratory until analysis. The samples were

ground to 0.5 mm flour particles prior to analysis.

Extraction and clean-up

The method of Shephard *et al.* (1990), with some modifications, was used for extraction of FB_1 and clean-up. A suspension was made of 25 g flour and 50 ml of a mixture of methanol and sodiumacetate buffer, 0.5 mol l^{-1} in water at pH 6.5, (75+25, (v/v)) and shaken on an orbital shaker for 1 hour. The suspension was filtered through a pre-folded paper filter. When the mass fraction was over 2,000 ng FB_1 g^{-1} maize, the filtrate was diluted with extraction solution and filtered once more.

Clean-up of the filtrate was carried out using an automatic sample preparation with extraction columns (ASPEC) system (Gilson) and 100 mg strong anion exchange columns (SAX, Varian). Each column was conditioned with 2 ml methanol and 2 ml of a mixture of distilled water and methanol (50+50 (v/v)), successively. An aliquot of 6 ml of the filtered extract was applied at a speed of 1.5 ml min^{-1}. The column was washed with 2 ml of the mixture of distilled water and methanol (50+50 (v/v)) and 1 ml methanol. FB_1 was eluted with 3 ml of a mixture of methanol-acetic acid (24+1 (v/v)), successively. The solution was evaporated to dryness under a stream of nitrogen at 60°C and the residue was stored at 4°C.

HPLC analysis

The residue was re-dissolved in 3 ml borate solution in water ($1x10^{-2}$ mol l^{-1}) and placed in the ASPEC. An aliquot of 200 µl of OPA-reagent (40 mg *o*-phthaldialdehyde in 5 ml borate solution, 1 ml methanol and 50 µl 2-mercaptoethanol) was transferred into a glass tube by the ASPEC and 800 µl of the re-dissolved residue was added. The solution was mixed by blowing air into the solution and one time sucking and draining of the whole volume. An aliquot of 10 µl was automatically injected on the HPLC column (C_{18} microsphere reversed phase, Chrompack, 3 µm particle size, 10 cm length and 4.6 µm ID). Reaction time, defined as the time between addition of re-dissolved residue to the OPA-reagent and injection, was 255 seconds. Fluorescence detection was at 335 and 450 nm excitation and emission wavelengths, respectively. A FB_1 standard from Sigma was

used to calculate the mass fraction of FB_1 in the sample. A standard of calibration solution of 0.4 µg FB_1 ml^{-1} was prepared in acetonitrile-water (50+50 (v/v)). An amount of FB_1, that gave a peak area equivalent to 400 ng FB_1 g^{-1} maize, was injected. Calibrants were injected at regular intervals after each three to four injections of extracts of test portions. The mass fraction of FB_1 in each test portion was determined by comparing the peak area of FB_1 in the test portion to the mean peak area of two adjacent injections of FB_1 calibrant. The results were corrected for the recovery on the experiment day.

Performance characteristics

Some performance characteristics were defined and some performance criteria were set for the modified method of analysis. The limit of determination was defined as 10 times the noise of a chromatogram of a test portion containing a trace amount of FB_1. The region of detection was between 3 and 10 times the noise. Mass fractions in this region are indicated as `trace` amount in Table 1.

Recovery experiments were carried out on several days over a three month period of time. At each experiment day, blank maize test portions were spiked in duplicate with FB_1 standard at a mass fraction of 200 ng FB_1 g^{-1}. The mean recovery was determined and the following analytical performance criteria were defined. The recovery (average of two) had to be > 60 % in each experiment with a coefficient of variation of $\leq$ 10 %.

For quality control purposes at each experiment day a 'laboratory internal reference material' (LIRM) was used. The FB_1 mass fraction in the LIRM was determined and corrected for recovery. The coefficient of variation of all LIRM analyses was required to be $\leq$ 10 %.

The concentration range of the HPLC system, where the detection of FB_1 mass fractions was linear, was determined. FB_1 standards were applied in duplicate in the range of 0.025 to 22 ng, equivalent to mass fractions of 3 to 2,800 ng FB_1 g^{-1} maize. The linear concentration range of the HPLC system was defined as the range of those FB_1 mass fractions of which the average experimentally found mass fraction deviated $\leq$ 20 % of the theoretically applied mass fraction.

Table 1. *Mass fractions of fumonisin B_1 in maize imported in The Netherlands.*

Year of sampling	Country of origin	Maize Char.[$]	Mass fraction ng fumonisin B_1 g^{-1}	Year of sampling	Country of origin	Maize Char.	Mass fraction ng fumonisin B_1 g^{-1}
1994				31	-		40
1	France	waxy	30	32	-		50
2	France		2,610	33	-		100
1995				34	France		100
3	France		trace[†]	35	-		100
4	-[‡]		30	36	France		100
5	France		100	37	Greece		100
6	-		170	38	Greece		170
7	France		210	39	France		210
8	France		220	40	-	waxy	270
9	Bahrain		250	41	France		280
10	-		300	42	-		290
11	France		320	43	-		520
12	-		410	44	-	waxy	540
13	France		640	45	Greece		560
14	France		650	46	-	waxy	590
15	France		660	47	-		600
16	France		790	48	France		630
17	France		820	49	-		630
18	France		1,020	50	-	waxy	650
19	France		1,110	51	-		690
20	France		1,200	52	-	waxy	740
21	-		1,240	53	-	waxy	760
22	France		1,270	54	-	waxy	850
23	France		2,760	55	-		860
24	France		3,350	56	-	waxy	870
1996				57	-	waxy	880
25	-		nd[*]	58	France		930
26	-		30	59	-		950
27	-	waxy	30	60	-		1,070
28	-		40	61	-	waxy	1,220
29	-		40	62	France		1,660
30	-		40				

[$] Characteristics of maize. Waxy maize or not known

[‡] Unknown country of origin

[*] Below limit of detection, 8 ng fumonisin B_1 g^{-1}

[†] In region of detection, 8-25 ng fumonisin B_1 g^{-1}

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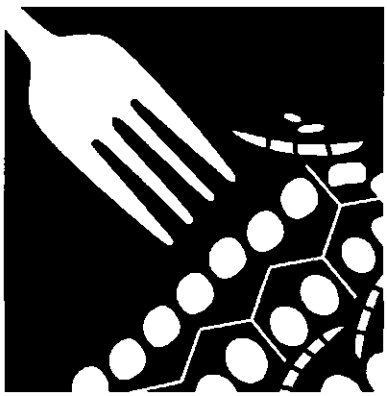
6

The occurrence of fumonisin B_1 in maize-containing foods in The Netherlands



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Food Addit. Contam. (1998) in press

ABSTRACT

Seventy-eight maize-containing foods obtained from retail stores in The Netherlands were analysed for fumonisin B_1 contamination. Fumonisin B_1 was detected in thirty-six percent of the samples in mass fractions ranging from 8 to 1,430 ng fumonisin B_1 g^{-1} maize. The mycotoxin was detected in forty-six percent of the minimal treated maize samples (n=39; maize for bread production, maize for popcorn, maize flour and polenta) in mass fractions ranging from 8 to 380 ng fumonisin B_1 g^{-1}. Twenty-six percent of the maize-containing processed foods (n=39; tostada, canned maize, maize starch, maize bread, popped maize, flour mixes, maize chips and cornflakes) was contaminated with fumonisin B_1 in mass fractions ranging from 8 to 1,430 ng fumonisin B_1 g^{-1}. This survey shows that maize-containing foods in The Netherlands can frequently be contaminated with fumonisin B_1.

INTRODUCTION

Fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium moniliforme,* has carcinogenic properties in experimental animals and, possibly, has been related to the aetiology of human oesophageal cancer *(2)*. Most reports on its occurrence deal

with maize *(10)*, although, incidentally its occurrence has been detected in naturally contaminated rice, sorghum and navy beans *(4, 7, 12)*. FB_1 is stable to heat, acid, dryness and salt and will thus resist normal food processing conditions *(5)*. The most important food ingredients that can be contaminated with FB_1 are maize and maize-containing foods *(1, 8)*.

This paper is part of an exposure assessment of humans in The Netherlands to FB_1. The occurrence of FB_1 was determined in maize imported in The Netherlands as described elsewhere *(6)*. FB_1 was detected in 98 % of 62 maize samples from lots intended for food production. Eighteen percent of these maize samples contained FB_1 in mass fractions over 1,000 ng FB_1 g^{-1}. The high incidence of contamination and the high contamination levels of the food raw materials requires attention for FB_1 contamination of maize-containing foods.

The results presented here give an overview of the fumonisin B_1 contamination of the most relevant maize-containing foods in The Netherlands.

MATERIALS AND METHODS

Samples

A total of 59 maize-containing food commodities was purchased in local retail stores in 1995. Nineteen samples of maize for bread production were obtained at milling factories in The Netherlands in 1996. The samples were refrigerated upon arrival in the laboratory and kept at 4°C until analysis.

The commodities were divided into two groups depending on the degree of processing. Group A was composed of 39 samples of minimal treated foods: maize for bread production and popcorn (categories 1 and 2), maize flour (category 3) and polenta (category 4). Group B was composed of processed maize-containing food commodities. Processes used were addition of water, heat treatment or water extraction, and maize-containing food commodities to which other ingredients were added such as other cereals, salt, sugar and spices. Commodities belonging to group B were: tostada (category 5), canned maize (category 6), maize starch (category 7), maize bread (category 8), popped maize (category 9), flour mixes (category 10), maize chips (category 11) and cornflakes (category 12).

The samples of maize for bread production and popcorn were ground to 0.5 mm flour prior to analysis. The samples of maize for popcorn intended for use in the microwave oven were first cleaned by melting and removing the fatty substance at 40°C. The samples of tostada, popped maize, maize chips and cornflakes were ground in a kitchen grinding apparatus. The samples of canned maize and maize bread were ground in a Waring blender. The samples of maize flour, polenta, maize starch, and flour mixes were not treated before analysis.

Extraction, clean-up and HPLC analysis

The LC method of Shephard *et al.* (1990) was used, with some modifications for extraction of FB_1, clean-up and HPLC. The procedure is based on anion-exchange clean-up and was described elsewhere *(6)*. If a test portion absorbed much fluid, the ratio g sample to ml extraction solution was decreased.

A FB_1 standard was obtained from Sigma. A calibrant solution of 0.4 µg FB_1 ml^{-1} was prepared in acetonitrile-water (50+50 (v/v)). Amounts of FB_1 were injected, that gave peak areas equivalent to 400 ng FB_1 g^{-1} maize. Calibrants were injected at regular intervals after each three to four injections of extracts of test portions. The mass fraction of FB_1 in each test portion was determined by comparing the peak area of FB_1 in the test portion to the mean peak area of two adjacent injections of FB_1 calibrant.

Determination of performance characteristics

The establishment of the limit of determination (25 ng FB_1 g^{-1} maize) and the region of detection (8 to 25 ng FB_1 g^{-1} maize) in maize have been described elsewhere *(6)*. Quality control was carried out on each experiment day to check if the procedure (extraction, clean-up, HPLC) performed according to pre-defined limits. The recoveries of FB_1 from blank samples, spiked in duplicate at 200 ng FB_1 g^{-1} maize, and the FB_1 mass fraction of a laboratory internal reference material (LIRM) (maize flour) were determined on each experiment day. The pre-defined ranges were 66 to 82 % and 60 to 70 ng FB_1 g^{-1} maize for the mean recovery and the FB_1 mass fraction in the LIRM, respectively *(6)*.

Table 1. *Fumonisin B_1 mass fractions of maize intended for bread production, category 1.*

Maize for bread production	
Sample nr.	Mass fraction ng fumonisin B_1 g^{-1}
1	nd*
2	nd
3	nd
4	nd
5	nd
6	nd
7	nd
8	nd
9	nd
10	nd
11	trace†
12	160
13	170
14	170
15	230
16	240
17	240
18	270
19	380
Recovery ng fumonisin B_1 g^{-1} (nr determinations): 69-70 (2)	

* Below limit of detection, 8 ng fumonisin B_1 g^{-1}

† In region of detection, 8-25 ng fumonisin B_1 g^{-1}

Table 2. *Fumonisin B_1 mass fractions of maize intended for popcorn, category 2.*

Maize for popcorn	
Sample nr.	Mass fraction ng fumonisin B_1 g^{-1}
1	nd*
2	nd
3	nd
4	nd
5	nd
6	nd
7	nd
8	nd
9	trace†
10	110
Recovery ng fumonisin B_1 g^{-1} (nr determinations): 70-71 (2)	

* Below limit of detection, 8 ng fumonisin B_1 g^{-1}

† In region of detection, 8-25 ng fumonisin B_1 g^{-1}

On each experiment day, the recovery was determined for each food commodity tested that day by spiking a test portion of that commodity in duplicate at 200 ng FB_1 g^{-1}. The recovery obtained for that commodity on the same experiment day was used for calculating the final FB_1 mass fraction in a sample.

RESULTS AND DISCUSSION

The results of the analysis of the most significant maize-containing food commodities regarding FB_1 contamination are presented in Tables 1 to 4. The recovery for each commodity is shown in the same tables. The results of this survey show that FB_1 can be frequently detected in maize-containing foods in The Netherlands in mass fractions below 500 ng fumonisin B_1 g^{-1}. Incidentally, higher contamination levels were found.

Table 3. *Fumonisin B_1 mass fractions of maize flour, category 3.*

Maize flour	
Sample nr.	Mass fraction ng fumonisin B_1 g^{-1}
1	nd*
2	nd
3	40
4	50
5	50
6	60
7	90
Recovery ng fumonisin B_1 g^{-1} (mean ± SD (nr determinations)) : 69±6 (9)	

* Below limit of detection, 8 ng fumonisin B_1 g^{-1}

The quality control parameters for the procedure, the mean recovery of FB_1 from spiked blank maize samples and the mass fraction of FB_1 in the LIRM, were 72 % (determined 22 times) and 64 ng FB_1 g^{-1} maize (determined 11 times), respectively.

These values were within the ranges of the pre-defined limits. The FB_1 recoveries obtained for the food commodities belonging to categories 1 to 4 (Tables 1 to 4), also met these pre-defined criteria. The recoveries for food commodities belonging to group B (categories 5 to 12, Table 4) on the average were lower and varied considerably, with lowest recoveries below 40 % for maize chips and cornflakes. Despite the low recoveries, the data give a good impression on FB_1 contamination in processed maize-containing food commodities belonging to group B.

Table 4. *Fumonisin B_1 mass fractions in various maize-containing foods, categories 4 to 12.*

Category	Sample	Pos./tot.[1]	Mass fraction ng fumonisin B_1 g^{-1} Range	Recovery Mean ± SD (nr. det.)[2] %
4	Polenta	2/3	nd*-40	71±8 (3)
5	Tostada	0/1	nd	70 (1)
6	Canned maize	0/6	nd	66±13 (5)
7	Maize starch	0/5	nd	62±12 (3)
8	Maize bread	1/2	nd-80	57–59 (2)
9	Popped maize§	3/5	nd-300	54±12 (6)
10	Flour mixes‡	2/6	nd-trace†	40±9 (10)
11	Maize chips	3/9	nd-160	36±17 (10)
12	Cornflakes	1/5	nd-1,430#	31±16 (8)
Total		12/42	nd-1,430	

[1] Ratio of number of positive samples to all samples

[2] Recovery, mean ± SD and, in brackets, number of determinations

* Below limit of detection, 8 ng fumonisin B_1 g^{-1}

† In region of detection, 8-25 ng fumonisin B_1 g^{-1}

§ Category of puffed maize, broken maize, maize flakes and waffles

‡ Including children's breakfast porridge

\# Recovery of 16 % for positive sample

The low and variable recoveries obtained for commodities 5 to 12 indicate that the method of analysis for FB_1 with anion exchange chromatography clean-up is

probably not the most suitable one for processed foods. When a detailed estimation of human exposure to FB_1 is required a more accurate method to determine FB_1 in processed foods or 24-hour duplicate diet samples should be developed.

A recent analytical study of 62 samples of maize lots imported in The Netherlands and intended for food production revealed 98 % of the samples was contamination with FB_1 at mass fractions up to 3,350 ng FB_1 g^{-1} *(6)*. The levels of FB_1 in these imported maize samples are much higher compared to the levels found in minimal treated commodities analysed in this study (categories 1 to 4). This apparent difference in FB_1 incidence and contamination level between maize and minimal treated or processed maize is also reported by other authors. High incidence and contamination levels were reported for FB_1 in non- or minimal treated maize (comparable to categories 1 to 4) with incidences of contamination over 60 % and mass fractions up to 16,000 ng FB_1 g^{-1} in polenta/semolina *(10)*. For processed maize (comparable to categories 5-12), reported incidences were below 50 % and FB_1 mass fractions were generally below 500 ng FB_1 g^{-1} *(3, 8, 10, 11)*. These differences might be the result of dilution of maize in food commodities, or may depend on the variations in maize cultivar or quality requirements for maize for the various destinations. It is unlikely that the low levels in minimal treated or processed foods are caused by instability of the mycotoxin during processing since it is known that FB_1 is stable under process conditions *(5)*.

The results of this survey show that fumonisin B_1 can be frequently detected in maize-containing foods in The Netherlands in mass fractions below 500 ng fumonisin B_1 g^{-1}, with one sample containing 1,430 ng fumonisin B_1 g^{-1}. As a consequence, the population might be continuously exposed to fumonisin B_1.

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7

Human exposure assessment to fumonisin B_1

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ABSTRACT

Fumonisin B_1 is currently regarded as most significant mycotoxin produced by *Fusarium*. It has carcinogenic properties and may play a role in the aetiology of human oesophageal cancer. The human population is exposed to fumonisin B_1 primarily through the intake of maize containing the mycotoxin. Maize consumed in The Netherlands is imported from all parts of the world. Since processing will not affect the mycotoxin, the fumonisin B_1 intake is directly related to the quantity of maize consumed. Literature results on the occurrence of fumonisin B_1 in a total of 349 samples of maize from 18 countries world-wide, demonstrated the presence of this mycotoxin in 93 % of the samples. The median fumonisin B_1 contamination of all samples was 420 ng fumonisin B_1 g^{-1} and the average contamination level was 1,359 ng fumonisin B_1 g^{-1} maize. Human intake of fumonisin B_1 was estimated based on the maize consumption of all people in The Netherlands in 1992. A probability distribution was derived, allowing the estimation of exposure of the population to fumonisin B_1 intake in relation to maize intake. It showed that from the group at risk, people with gluten intolerance such as people with celiac or Dühring's disease, 37 % is estimated to be daily exposed to an intake of $\geq 10^5$ ng fumonisin B_1

and 97 % to levels $\geq 10^3$ ng fumonisin B_1 per person. For all people in The Netherlands these percentages would be 1 % and 49 %, respectively.

INTRODUCTION

Risk assessment studies are used to estimate the probability that harm to the health of the consumer may occur following exposure to a certain hazardous agent. It is a four step process comprising hazard identification, exposure assessment, hazard characterisation (including dose-response assessment) and risk characterisation. The risk assessment provides quantitative information on factors leading to a certain unwanted situation. This information is essential in managing the unacceptable situation (15).

The hazard identification for *Fusarium* mycotoxins has recently been described elsewhere *(26)*. That study revealed that several species of the genus *Fusarium* excrete mycotoxins during the field period after the crop (cereals, vegetables and fruits) is infected by the fungus. Infection of crops with *Fusarium* and its mycotoxins can be symptom-free *(34)*. The mycotoxins that are present in the crop at harvest are not removed by processing the raw material for food production, e.g., by changes in temperature, pH and salt concentration *(23)*.

The effects of *Fusarium* mycotoxins on human health can vary considerably between the mycotoxins. Zearalenone has oestrogenic effects on humans, while ingestion of large amounts of deoxynivalenol (vomitoxin), acetyldeoxynivalenol, nivalenol or T-2 toxin can result in acute food poisoning, vomiting, weight loss and even human casualties *(3, 29, 42)*. Fumonisin B_1 (FB_1) is, however, currently regarded as the most important *Fusarium* mycotoxin since it is carcinogenic and possibly playing a role in the aetiology of human oesophageal cancer *(7, 16, 37)*.

The exposure assessment of humans in The Netherlands to FB_1 is described in this paper. Exposure assessment can be defined as the qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant *(9)*. In case of FB_1 it requires knowledge on the route of exposure, quantification of the mycotoxin in food and information on the intake of foods containing FB_1. In addition, the groups

in the population that are at risk for high intake of FB_1 are identified.

MATERIALS AND METHODS

A selection from the relevant scientific literature was reviewed for *Fusarium* species that were able to produce FB_1. Food raw materials were identified in which the mycotoxin was detected. The route of exposure of the population in The Netherlands to these food raw materials and, thus, to FB_1 was determined. A survey on geographic origin of the food raw materials was included.

The literature was reviewed for published results on the occurrence of FB_1 in maize. In order to obtain random data, only those papers were selected in which the concentration of FB_1 per sample was presented. Part of the data from an experiment carried out by the authors on the occurrence of FB_1 in maize imported in The Netherlands and intended for food production was included *(27)*. Data of samples related to intoxications in animals or obtained from areas in which the incidence of oesophageal cancer was high were not used. Likewise, samples referring to mouldy or 'unhealthy' maize were excluded from the data set.

Table 1. Fusarium *species producing fumonisin B_1*.

Fusarium species	Isolation source	
F. moniliforme	Maize	*(16)*
F. anthophilum	Maize	*(25)*
F. dlamini	Maize	*(25)*
F. napiforme	Maize	*(25)*
F. nygamai	Maize	*(25)*
F. proliferatum	Maize	*(25)*
F. subglutinans	Maize	*(39)*
F. oxysporum var. *redolens*	*Pinus strobus* L. (Eastern white pine)	*(2)*
F. polyphialidicum	*Pinus strobus* L.	*(1)*

Data on the consumption of maize by the population in The Netherlands (not including canned maize) were obtained from import and export data and from

reports prepared by van Dooren-Flipsen *et al.* (1995, 1996). The latter studies are based on data collected in the 'Voedsel consumptiepeiling 1992' (VCP) study from 1992 in The Netherlands *(40)*. Data on maize consumption in Canada and the European Union were used for comparison *(20, 33)*. Groups in the population reporting the consumption of maize in the Canadian study were identified as 'eaters only' and their intake of maize was estimated *(20)*. The intake ratio of the group of 'eaters only' to all people in the Canadian study was used to estimate the intake of maize by the group of 'eaters only' in The Netherlands and the European Union.

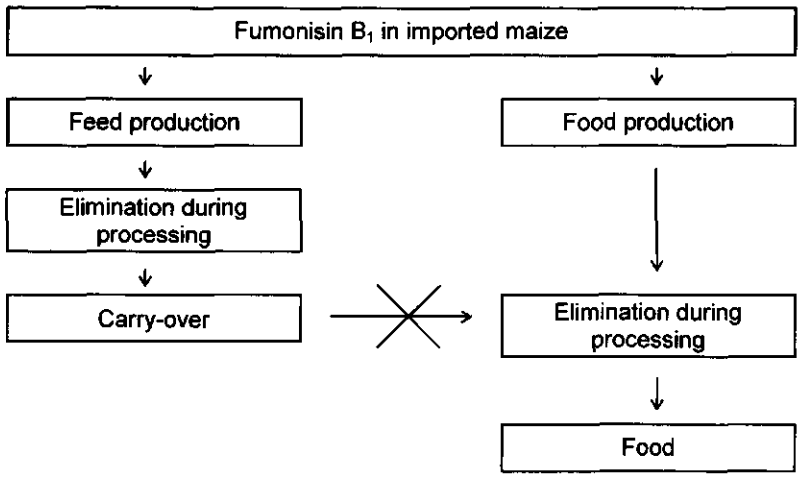

Figure 1. *Exposure route of people in The Netherlands to fumonisin B_1.*

To generalise the literature data on FB_1 contamination, these literature levels were fitted to some common statistical probability distributions. The logarithms of the FB_1 levels fitted reasonably well with a normal distribution, when using the mean and standard deviation of the experimental samples. The total amount of FB_1 ingested by the population depends on the amount of maize consumed and the concentration of FB_1 in the maize consumed. Given a consumption of y g maize per day, the probability of an intake of more than x ng FB_1 per day equals the probability of randomly selecting a maize sample that contains more than x/y ng FB_1 per g maize. In a more formal notation: P (intake > x ng FB_1 per person per day | Consumption = y g maize) = P (Maize sample containing > x/y ng FB_1 per g maize).

RESULTS

FB_1 was first detected in maize in South Africa by Gelderblom *et al.* (1988). Since then, it has world-wide been detected in maize. Recently, the mycotoxin was detected in naturally contaminated rice, sorghum and navy beans *(22, 28, 38)*.

A total of nine *Fusarium* species that could produce FB_1 under laboratory conditions was found in the literature, most often isolated from maize (Table 1). Additionally, FB_1 production was also reported by the fungus *Alternaria alternata* f.sp. *lycopersici*, a host specific pathogen of tomato plants *(6)*.

Table 2. *Countries of origin or marketing of maize imported in The Netherlands in 1994, 1995 and 1996 (5).*

Country of origin or marketing	Weight percentage of total (average of three years)
France	80
Germany	10
Belgium and Luxembourg	4
The United States	3
Argentina	1
Canada, Denmark, Hungary, Ireland, Italy, Portugal, Republic of South Africa, Slovakia, Spain, Thailand, United Kingdom	0.01-1
Austria, Egypt, Gambia, Ghana, Indonesia, Israel, Kenya, Malaysia, Mexico, Peru, Sudan, Surinam, Swaziland, Switzerland, Turkey, Uganda, Zimbabwe	<0.01

The route of exposure of the population in The Netherlands to FB_1 is shown in Fig. 1. All maize for food production and most maize for feed production are imported in the country. The mycotoxin is stable under processing conditions and it can be determined in foods and feed that contain maize *(23)*. FB_1 can be hydrolysed to the aminopentol when strong alkaline solutions are used, such as in the process of nixtamalisation of maize *(18, 32)*. However, the resulting products are still regarded as toxic compounds *(23)*. FB_1 is water-soluble and can be partly washed out in the process of starch isolation. It can accumulate in various end products *(4)*. Carry-over of FB_1 through animal tissue or milk has not been reported *(30)*.

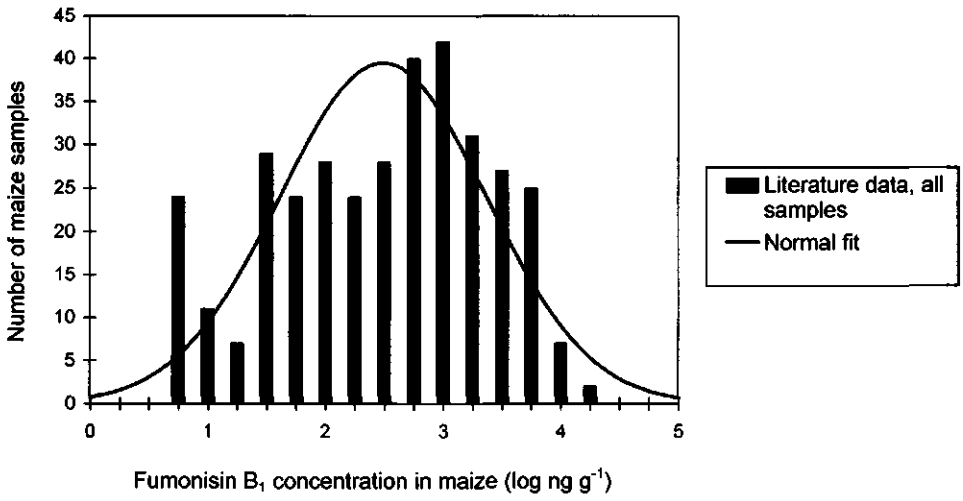

Figure 2. *Frequency distribution of fumonisin B_1 concentrations in maize samples. Data from literature in classes around the indicated midpoints are represented by the bars. The line represents the best fit normal distribution.*

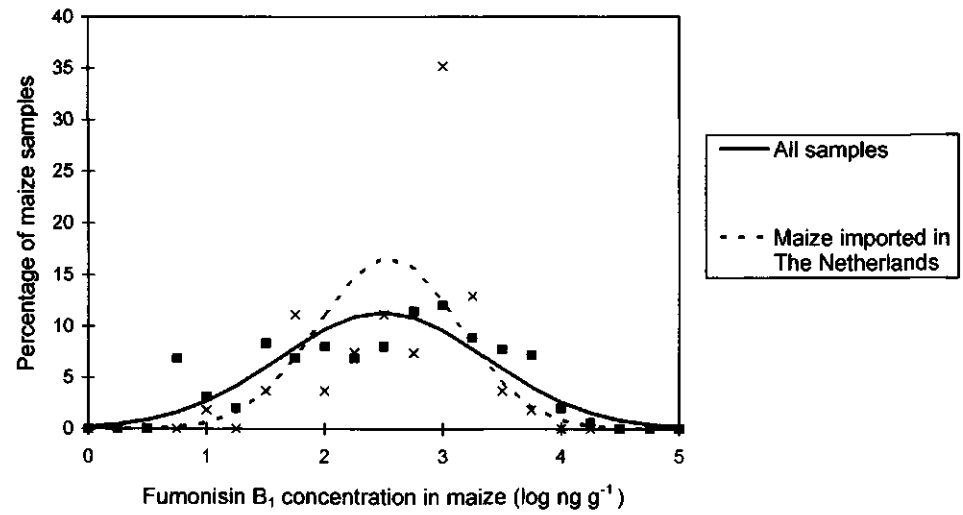

Figure 3. *Frequency distribution of fumonisin B_1 concentrations in two different groups of maize samples. Normal distribution fitting to the data of the log fumonisin B_1 concentration in maize imported in The Netherlands (- - - -) and from all samples (——) with corresponding data points x and ■, respectively.*

The annual net amount of maize imported in The Netherlands and intended for both food and feed production, in the years 1994, 1995 and 1996 was 1,668,489 ton on average *(5)*. Of this, 43,333 ton, 2.6 %, was intended for human consumption *(21)*. The countries that market the maize are presented in Table 2.

Table 3. *Fumonisin B_1 contamination of maize of various geographic regions.*

Continent marketed	Year of sampl.[1]	Number pos./total[2]	Concentration ng fumonisin B_1 g^{-1}			Median all	Limit det.[5]	
			Range	Avg. pos.[3]	Avg. all[4]			
Africa	1994	34/37	nd[†]-1,910	236	217	105	20	*(10)*
	1989-1992	29/31	nd-2,630	282	263	80	10	*(11)*
Asia	1992	8/9	nd-1,450	794	706	740	50	*(41)*
Europe	1989-1992	51/67	nd-2,330	382	291	30	10	*(11)*
	1992-1993	8/8	100-5,310	2,899	2,899	2,920	10	*(12)*
	1994-1996	53/54	nd-3,353	676	663	615	25	*(27)*
North America	1990-1996	70/70	36-2,940	703	703	542	20	*(35)*
South America	1991	17/17	1,110-6,695	2,876	2,876	2,385	50	*(36)*
	1990-1991	47/48	nd-18,520	5,491	5,376	5,065	nr[‡]	*(17)*
	nr	8/8	85-8,791	2,131	2,131	410	20	*(8)*
Total		325/349	nd-18,520	1,459	1,359	420		

[1] = Year of sampling

[2] = Number of positive samples/total number of samples

[3] = Average concentration of positive samples

[4] = Average concentration of all samples, with 0 ng fumonisin B_1 g^{-1} taken for the samples below limit of determination

[5] = Limit of determination

[†] = Below limit of determination

[‡] = Not reported

Published data from nine studies on the occurrence of FB_1 in maize, revealed a total of 349 maize samples. The maize samples originated from 18 countries world-wide. Thirty-two percent of the samples were contaminated at levels over 1,000 ng

FB_1 g^{-1} maize. The level of contamination varied between below limit of determination (10-50 ng FB_1 g^{-1} maize) and 18,520 ng FB_1 g^{-1} maize, with a median level of 420 ng FB_1 g^{-1} maize. The average contamination level was 1,359 ng FB_1 g^{-1} maize for all samples and 1,459 ng FB_1 g^{-1} maize for the positive samples (the FB_1 concentration was set at 0 for samples containing FB_1 below the limit of determination). The data include analytical results from 54 samples obtained from maize imported in The Netherlands *(27)*. Some characteristics of the data are presented in Table 3.

Taking the logarithms of the FB_1 concentrations in the above mentioned maize samples, a normal distribution with identical mean and standard deviation as the experimental data was the best fit through the data. In samples containing the mycotoxin in amounts below the limit of determination, the FB_1 concentration was set at 6.7 ng FB_1 g^{-1} maize to allow the best fit. This concentration was based on the smallest deviation of the normal distribution using the least squares method. This gave a reasonable fit, as is shown in Fig. 2.

Fig. 3 shows that the distribution of FB_1 in maize imported in The Netherlands (n=54) had identical distribution to maize from all parts of the world. The lognormal distribution of FB_1 in the imported maize is shown together with the lognormal distribution of FB_1 in all maize samples (the latter is identical to Fig. 2).

The intake of maize by the various groups of the population in The Netherlands, European Union and Canada is presented in Table 4. It should be taken into consideration that the actual amount of daily maize intake by individuals from the group can deviate from the average. The VCP study carried out in The Netherlands in 1992 was based on a group of 6,218 people, age 1 to 92, belonging to 2,475 households *(40)*. Canned maize was not included. The study conducted in Canada in 1970-72 reflects the amount (g) of maize, dry maize, meal, flour and semolina consumed by adults per day *(20)*. A study carried out in the European Union concluded that the amount of maize consumed by an adult person in the European Union is 4.2 % of the amount of wheat consumed, which was 0.171 kg per person per day *(33)*. The weight of an adult in The Netherlands in 1992 was 68 and 80 kg for females and males, respectively, with an average of 74 kg *(40)*. The average

weight for the people in the Canadian study was 60 kg *(20)*.

The maize consumption ratio `eaters only` to all people was estimated in the Canadian study *(20)*. This ratio is used to calculate the amount of maize consumed by the `eaters only` groups in the other two studies. The group of `eaters only` consumes 14 times more maize than the people at average.

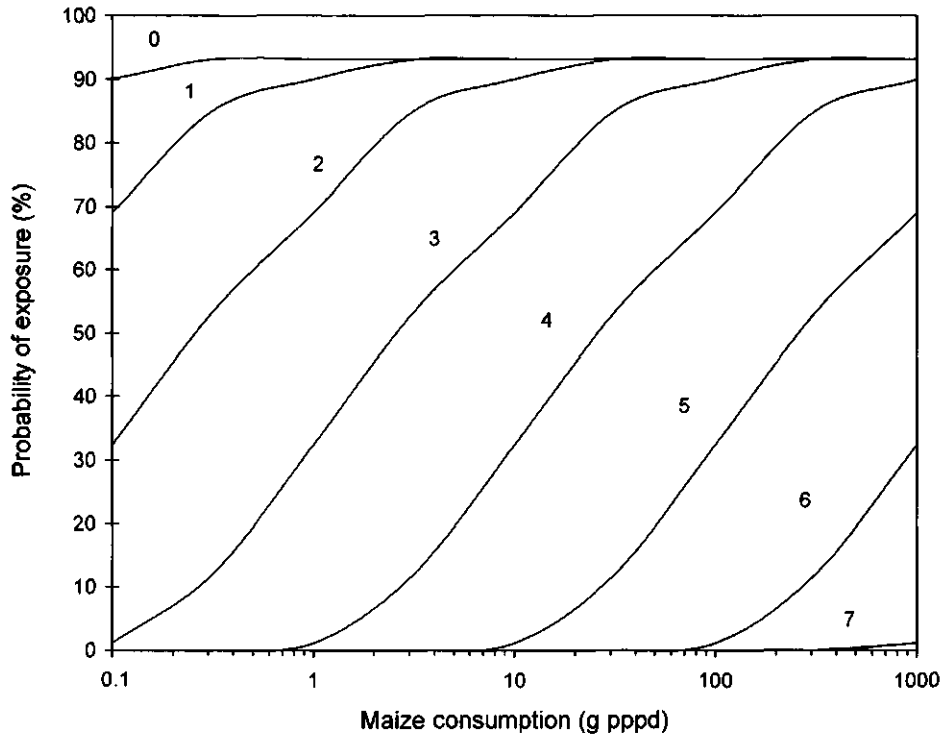

Figure 4. *Iso-lines of daily exposure to fumonisin B_1 levels, based on literature data. The iso-lines show the probability of being exposed to fumonisin B_1 levels higher than the indicated fumonisin B_1 amount (ng fumonisin B_1 per person per day with average body weight of 74 kg), given a certain consumption of maize (g maize per person per day). The numbers above the iso-lines indicate the log_{10} units of fumonisin B_1 intake in ng per person per day.*

The group of people with gluten intolerance, people with celiac or Dühring's disease, are included in this paper as the group at risk. They consume a gluten-free diet in which all wheat, barley, oats and rye is replaced with maize or rice. The amount of maize consumed by this group was estimated by substitution of all the wheat (125 g per person per day (pppd)), barley (5 g pppd), oats (27 g pppd), rye (1 g pppd) and other cereals (1 g pppd) consumed by the population in The Netherlands with maize *(40)*. The number of people with celiac disease is approximately 3,500, 0.02 % of the population in The Netherlands *(24)*.

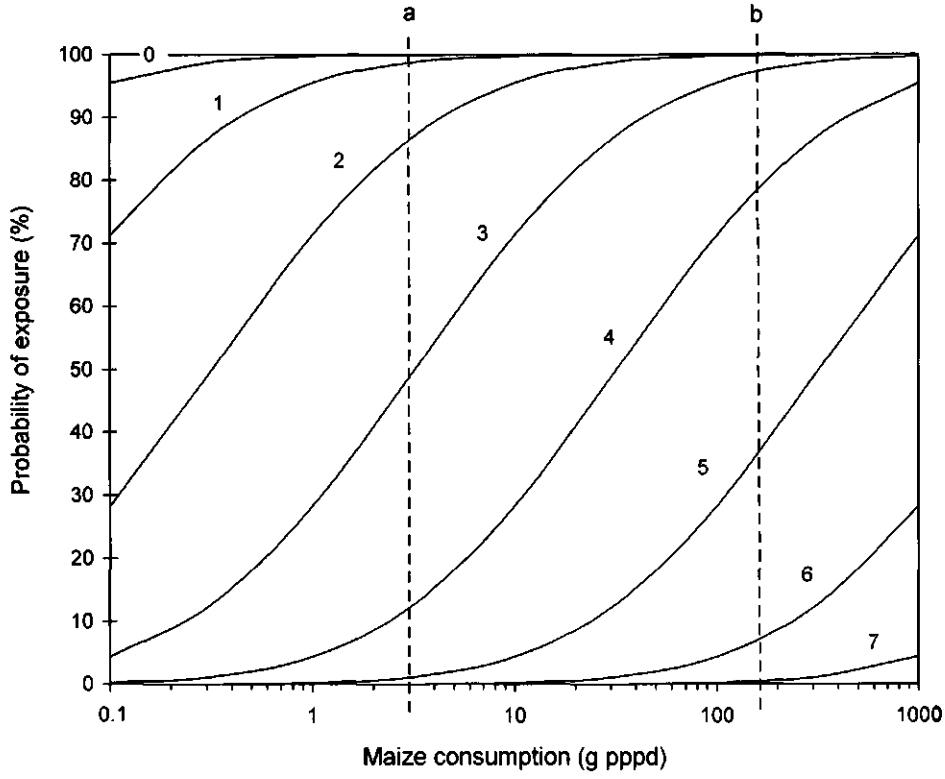

Figure 5. *Iso-lines of daily exposure to fumonisin B_1 levels, based on lognormal fit data. The iso-lines show the probability of being exposed to fumonisin B_1 levels higher than the indicated fumonisin B_1 amount (ng fumonisin B_1 per person per day with an average body weight of 74 kg), given a certain consumption of maize (g maize per person per day). The numbers above the iso-lines indicate the log_{10} units of fumonisin B_1 intake in ng per person per day. Dotted lines indicate the average maize consumption (g maize per person per day) by all people in The Netherlands (line a) and by the people from the group at risk (line b).*

The estimated part of the population (%) exposed to various amounts of FB_1 per day, depending on maize consumption, is shown in Fig. 4 and 5. These Figures show the results for both the literature data (Fig. 4) and the normal fit through these data (Fig. 5). In these Figures, the lognormal distribution (exposure levels) is shown for 7 levels of FB_1 intake, in ng FB_1 per person per day. It should be taken into consideration that the average weight of people from different countries can vary.

Table 4. *Daily intake of maize by populations in various regions of the world and by people belonging to the group at risk. Mean value in g per person, per day.*

Country or group of people	Mean daily maize intake (g)		Survey year		
	All people	Eaters only			
The Netherlands	3.0	*42*[*‡]	1992	Population	*(14)*
European Union	*7.2*[§]	*99*[‡]	1994	Population	*(33)*
Canada	2.6	36	1970-72	Adults	*(20)*
People with celiac or Dühring's disease	*162*	-	1992	Population	

* Data printed in italics are calculated, based on assumptions
‡ Calculated, assuming ratio all eaters to 'eaters only' as in Canadian survey
§ Calculated from wheat consumption
- All people in survey are considered eaters

Fig. 5 provides information for risk management. Indicated in dotted lines in Fig. 5 are the amounts of maize consumed on average by the population in The Netherlands, line a (3 g maize), and by the group at risk, line b (162 g maize). Depending on the tolerable daily intake per person (TDI_{pp}, ng FB_1 per person per day), the probability of a certain amount of the population being exposed to that FB_1 intake on a daily base can be derived from Fig. 5. For example, the TDI_{pp} for FB_1 in The Netherlands could be set at 1,000 ng FB_1. This is identical to the tolerable daily intake as set by the Joint FAO/WHO Expert Committee on Food Additives for

ochratoxin A intake *(19)*. The iso-line of 1,000 ng FB_1 (iso-line 3), the hypothetical TDI_{pp}, in Fig. 5 shows that at the before mentioned intakes of maize, 49 % of the total population and 97 % of the people from the group at risk are exposed to FB_1 at that level. If the TDI_{pp} would be set at $1x10^5$ ng FB_1, the percentages would be 1 % and 37 % for all people and people belonging to the group at risk, respectively. In case of a TDI_{pp} set at 1,000 ng FB_1, the concentration of FB_1 in the 3 g maize consumed on average by a person in The Netherlands (Table 4) would be 333 ng FB_1 g^{-1} maize. Likewise, this would be 6 ng FB_1 g^{-1} maize for the 162 g maize consumed by a person in the group at risk, well below the limit of determination. It must be emphasised that the here presented TDI_{pp} levels for FB_1 intake are for illustration only. The TDI_{pp} for FB_1 can only be determined when scientific data on toxicity of FB_1 in test animals becomes available.

DISCUSSION

FB_1 can be produced by at least nine species of the fungal genus *Fusarium* and the mycotoxin can be detected in maize, rice, sorghum and navy beans. Maize is the most important food raw material since the consumption of maize, containing FB_1, is possibly related to the aetiology of oesophageal cancer. Maize for food production is imported in The Netherlands, primarily from other European countries, but distribution of FB_1 does not dependent on the geographical origin of the maize. Processing the raw material for food production does not influence the FB_1 contamination. Therefore, the data on FB_1 contamination of maize world-wide were used to calculate intake of FB_1 by the population in The Netherlands.

A reliable and up-to-date set of data on the consumption of the commodity under investigation is important in the risk assessment. Regarding the data on import of maize intended for food production, the estimated intake of maize by the population in The Netherlands would be 8 g per person per day (population of 15 million). These data were not used in the here presented study since it is unknown which part of that maize is exported again, possibly as food commodity. To allow the estimation in the variation in the intake of FB_1 by the population, the size of the group of `eaters only` and the amount of maize consumed on average by this group should be

determined for the situation in The Netherlands. Additionally, a clear insight is required in how the data on consumption were obtained and handled and which assumptions were made. For example, van Dooren-Flipsen *et al.* (1995, 1996) assumed that all the modified starch present in soups, snacks and sauces, was wheat starch. In reality, a large part of this starch might by maize starch and, thus, the intake of maize by the Netherlands population could be underestimated. Data on intake of maize by the human population in Canada were derived from a study carried out in 1970-1972. If maize intake has changed over the years, the risks caused by FB_1 intake could be over- or underestimated. Geographical differences in consumption patterns can influence the results, as is shown in the study of Smith *et al.* (1994), as well as average weight per person in the group under investigation. The real intake of maize per day by the population in The Netherlands was estimated to be 2.4 % of the wheat intake, which is almost two times lower than the assumption of 4.2 % by Smith *et al.* (1996).

When assessing the hazards to the health caused by FB_1 intake, it is important to identify groups at risk in the population in The Netherlands. These are groups with high intake of maize and groups exposed to maize with high FB_1 content. People with gluten intolerance, such as people with celiac or Dühring's disease, consume considerable amounts of maize daily and are at risk for relatively high intake of FB_1. No specific group can be identified at risk for consumption of maize containing high amounts of FB_1 since all maize for human consumption is imported in The Netherlands and quality control can be implemented. FB_1 can be detected in beer brewed from maize *(31)*. However, the damage caused by the alcohol is regarded more significant to tumour induction and promotion in this group.

Almost half of the population in The Netherlands can probably be exposed daily to an intake of 1,000 ng fumonisin B_1 per person, based on the average maize intake of 3 g per person per day. Virtually all people in the group at risk, people with gluten intolerance such as people with celiac or Dühring's disease, can be exposed to levels of over 1,000 ng fumonisin B_1 per person per day, due to the high intake of maize by this group.

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8

Effects of low doses of fumonisin B_1 on kidney, liver and immune parameters in rats in a 28-day toxicity study

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ABSTRACT

Fumonisin B_1 has been associated with the aetiology of human oesophageal cancer, pig pulmonary oedema and equine leukoencephalomalacia. A 28-day toxicity study was carried out to increase knowledge on the target organs and toxic effects of low doses of fumonisin B_1, with special emphasis on several immunotoxic parameters. Four groups of 10 rats were dosed daily by gavage with 0 (control group), 0.19 (low dose group), 0.75 (mid dose group) and 3 (high dose group) mg fumonisin B_1 kg^{-1} body weight. Treatment with fumonisin B_1 did not affect body weight. Kidney weights of animals in the high dose group were statistically significantly reduced. Extensive apoptosis was observed in the medulla of the kidneys of animals in the mid and high dose treatment groups. Basophilic tubules and mitosis were indicative for the ongoing regeneration process. Sphinganine-to-sphingosine ratios in kidneys of animals in all groups were statistically significantly increased. γ-Glutamyltransferase levels in serum and sphinganine levels in liver tissue of animals of the mid and high dose groups were statistically significantly increased. No changes were observed in haematology, serum urea and creatinine concentrations or sphinganine and sphingosine levels or their ratio in serum. Immunoglobulin concentrations, mitogen

B.V., Woerden, The Netherlands) were provided *ad libitum*. A light-dark regime of 12 hours was maintained. Temperature and relative humidity were maintained at 20-24°C and 45-65 %, respectively.

Experimental procedure

Four groups of 10 animals were treated with FB_1 by daily intubation of 1 ml test solution per 100 g body weight for 28 consecutive days. The control, low, mid and high dose groups were exposed to 0, 0.19, 0.75 or 3 mg FB_1 kg^{-1} body weight per day, respectively. Treatment started on day 0 for animals numbered 1 to 5, 11 to 15, 21 to 25 and 31 to 35. Treatment of animals numbered 6 to 10, 16 to 20, 26 to 30 and 36 to 40 started on day 1. Clinical signs and mortality were checked daily. Body weight was recorded daily and food consumption twice per week. Intake of water was not recorded.

The animals were autopsied one day after the last treatment day on days 28 and 29. Blood was collected for haematology on the day of sacrifice. After exsanguination (abdominal aorta under ether anaesthesia) the organs were examined and sampled. The organs were weighed (see below) and grossly examined for macroscopic pathological changes.

Organ weights and histopathology

The following organs were examined macroscopically and fixed in 4 % neutral buffered formaldehyde: adrenals, brain, femural bone marrow, heart, kidney, liver, lung, lymph nodes (mandibular, mesenteric, popliteal), oesophagus, pituitary gland, spleen, stomach, testis, thymus, thyroid gland and small and large intestines (duodenum, jejunum, ileum, caecum, colon, rectum). Organ weights were determined except for femural bone marrow, pituitary gland and gastrointestinal tract. Part of the spleen, thymus and mesenteric lymph node and the left popliteal and mandibular lymph nodes were quickly deep frozen in liquid nitrogen. Tissue was processed for light microscopy as described previously *(30)*. The tissues were trimmed after fixation, embedded in paraffin and sectioned at 5 µm. Histological samples were prepared routinely after paraffin embedding and hematolin and eosin

(H&E) staining of 5 µm sections. When indicated by the histopathological results additional cuts were prepared. Apoptotic cells were detected by immunohistochemic staining using the '*In situ* cell death detection kit, POD' of Boehringer (Mannheim, FRG). Histopathology was documented using the PATHOS data-entry and reporting system (Pathology Operating Systems Ltd, Harrogate, England) or manually. All tissues of the high dose group and the control group were investigated for histopathology. In addition, the kidneys of animals from the low dose and the mid dose groups were histologically evaluated.

Haematology

Haematological parameters were determined using H1-E multispecies haematology analyser (Technicon, Miles Inc., Tarrytown, USA) according to the instructions of the manufacturer. The following haematological parameters were measured: leukocytes (WBC), erythrocytes (RBC), haemoglobin (HGB), haematocrit (HCT), mean cell volume of erythrocytes (MCV), platelets (PLT) and mean platelet volume (MPV). The following parameters were calculated: mean HGB mass of erythrocytes (MCH), mean HGB concentration of erythrocytes (MCHC), red cell distribution width (RDW) and HGB distribution in erythrocytes (HDW). Relative differential leukocytes were measured and calculated to absolute amounts (neutrophile granulocytes (Neut), lymphocytes (Lymph), monocytes (Mono), eosinophilic granulocytes (Eos), basophilic granulocytes (Baso), large unstained cells (Luc)). Femural bone marrow cytospin preparations were prepared as described elsewhere *(13)*.

Biochemistry

(i) Activity of liver enzymes and concentration of kidney parameters were determined in the sera according to the methods recommended by the manufacturer of the test kits (Roche Diagnostica, Brussels, Belgium). Enzyme activities measured were: alanine transaminase (ALAT) (Unimate-5 ALT), aspartate transaminase (ASAT) (Unimate-5 AST), γ-glutamyl transferase (γ-GT) (Unimate-3 GGT) and expressed as units l^{-1}. Concentrations of urea (Unimate-5 UREA) and creatinine (creat) (Unimate-7 CREA) were determined and expressed as mM and µM, respectively.

(ii) Sphingolipid analyses in serum and liver and kidney tissues, were performed at the MRC according to Riley *et al.* (1994b). Results were expressed as nM in serum and pmol mg^{-1} in tissues. Liver and kidney tissue samples were quickly deep-frozen in liquid nitrogen at autopsy and stored at -70°C prior to sphingolipid analyses. The serum and tissue samples (homogenised in 0.05 M potassium phosphate buffer) were shipped to MRC in South Africa on dry-ice.

Immunological parameters

(i) Serum immunoglobulin parameters were determined as described elsewhere, with some adjustments *(26)*. Anti-antibodies used were: IgG and IgA (purified at RIVM), IgM (Nordic, Tilburg, The Netherlands) and IgE (Sanbio, Uden, The Netherlands). The substrate, tetramethylbenzidine (TMB), was dissolved in citrate acetate buffer (0.11 M) at pH 5.5. The reaction was stopped after 10 min with 2 M sulfuric acid (H_2SO_4). The plates were read at 450 nm. The immunoglobulin concentrations for the individual animals were determined as a percentage of the concentrations in the pooled sera of the animals of the control group.

(ii) Mitogen responsiveness of lymphoid spleen cells was determined as described elsewhere *(13)*, using four dilutions of each cell suspension, analysed in triplicate. Mitogens used: concanavalin A (ConA) (Acros Organics, Geel, Belgium), phytohaemagglutinin (PHA) (Murex, Chatillon, France) and *E. coli* lipopolysaccharide (LPS) (Difco, Detroit, USA). Results were expressed as cpm (counts per minute) per culture (blanks subtracted).

(iii) Natural killer cell activity was measured as described elsewhere *(12)*, corrected for spontaneous and maximal ^{51}Chromium release and expressed as percentage of specific release. Four ratios of effect to target cells (E:T) were determined (25, 50, 100 and 200).

(iv). The fluorescence activated cell scan (FACScan) analysis of subpopulations of splenic lymphocytes were conducted as described previously *(30)*. Monoclonal antibodies MARK-1 (Sanbio, Uden, The Netherlands), ER-2, OX-8 and OX-19 (Serotec, Oxford, UK), all conjugated with fluorescein isothiocyanate (FITC), were used to identify B-cells, $CD4^+$-cells, $CD8^+$-cells and $CD3^+$-cells, respectively. The

results were expressed as percentages of cells and absolute number of cells per spleen.

Statistics

Histological findings were analysed statistically with the Fisher's exact probability test (two sided). All other results were tested by analysis of variance (ANOVA, single-factor), including differences between individual groups. *P*-values equal or smaller to 0.05 were considered statistically significant.

Table 1. *Effect of fumonisin B_1 treatment on body weight (g) and body weight gain (g) in the 4 weeks of exposure of rats (Mean ± SD, n=10 per group).*

	Control	Low dose	Mid dose	High dose	P*
week -1	72±16	73±14	75±14	71±15	0.9494
day 0	106±21	106±15	108±15	106±16	0.9882
week 1	148±23	152±16	153±16	148±19	0.8787
week 2	192±24	199±19	200±19	192±24	0.7280
week 3	236±28	247±19	248±20	238±27	0.6157
week 4	278±30	290±18	288±19	279±33	0.5904
Body weight gain	172±18	184±14	180±14	173±21	0.3179

*P-value of 1-factor ANOVA

RESULTS

In this 28-day study on toxicity of low doses of FB_1 in rats, including immune function tests, FB_1 was dosed at 0, 0.19, 0.75 and 3.0 mg FB_1 kg^{-1} body weight, which is equivalent to 0, 1.9, 7.5 and 30 mg FB_1 kg^{-1} feed (assuming that an amount of feed equal to 10 % of the total body weight was consumed daily by the rats).

No statistically significantly variations were observed between animals of the treatment groups for body weight or body weight gain in the 28-day period (Table 1). Neither feed intake nor feed conversion rate showed statistically significantly

changes between the groups (data not shown). The effects of FB_1 administration on organ weights are shown in Table 2. The absolute and relative weights of the kidneys of animals belonging to the high dose group were statistically significantly lower than the weights of kidneys of animals belonging to the control, low and mid dose groups (11 % as compared to the control group). No statistically significantly differences were observed in haematological parameters, leukocyte counts and femural bone marrow (Table 3).

The major histopathological effect of FB_1 was observed in the kidney (Table 4). Basophilic proximal tubules in the *pars recta* (outer medulla) accompanied by the presence of death cells were observed microscopically in the kidneys of animals in all three treatment groups (Fig. 1). In the mid and high dose groups, these basophilic tubules comprised almost all tubules in the outer stripe of the outer medulla. The regenerative character of the basophilic tubules was confirmed by the presence of mitotic cells. The observed tubular cell death was present as single death cells primarily in the lumen of basophilic tubules, but not in all basophilic tubules. Immunohistochemistry showed that the observed cell death was due to apoptosis (Fig. 2), incidentally extended in the cortex for animals which scored severe for basophilic tubules. In these cortical basophilic tubules, death tubular cells were incidentally present. Both for the presence of basophilic tubules and the presence of death cells a dose-response relationship could be observed. The low dose treatment animals had a score of basophilic tubules presence in the range of control animals. However, in these low dose treatment animals basophilic tubules could be observed both in the cortex and the outer medulla including death cells while in control animals basophilic tubules were only present in the cortex and death cells were absent. The observed interstitial nephritis which accompanied the basophilic tubules was minimal. As it was observed in treated animals the occurrence could be ascribed to the FB_1 treatment. Evaluation of the grade of the lesions, however, resulted in a highly statistically significantly difference between control and treated animals. Statistically significantly increases (Fisher's Exact Test) with $P=0.00036$ and $P<0.00001$ were observed for basophilic tubules score severe in the mid and high dose groups, respectively. The presence of death tubular cells was statistically

different (Fisher's Exact Test) for all treatment groups with P=0.01625, P=0.0001 and P<0.00001 for animals in the low, mid and high dose groups, respectively.

Table 2. *Effect of fumonisin B_1 treatment on absolute organ weights (mg) of rats (Mean±SD). In brackets the number of animals if not 10.*

	Control	Low dose	Mid dose	High dose	P*
Brain	1768±80	1807±77	1789±65	1752±47	0.3117
Pituitary	8±2	9±1	9±1	8±2	0.5098
Salivary gland	145±18	134±21	141±19	142±16	0.6311
Heart	1104±134	1081±62	1121±99	1081±83	0.7670
Lung	1850±466	2156±509	1794±289	1917±488	0.2987
Liver	12088±1709	12880±1584	12721±944 (9)	12014±1595	0.4816
Kidneys	2201±225	2332±218	2317±201	1968±233[a]	0.0023
Adrenals	42±4	44±6	45±3	42±4	0.4910
Testes	2763±335	2821±219	2892±279	2823±275	0.7858
Thymus	690±191	722±135	777±102	666±139	0.3701
Spleen	566±81	598±34	598±68	564±85	0.5369
Mes. Lymph nodes	349±93	356±57	329±38	352±58	0.7986
Popl. Lymph nodes	10±3	12±3	11±3	12±3	0.5296

* P-value of 1-factor ANOVA

[a] Statistically significantly different from control, low and mid dose groups (P<0.05)

Pyelitis was observed in two animals of the mid dose group accompanied by hyperplasia and inflammation of the urothelium. Urothelial hydropic degeneration occurred in one of these animals. As pyelitis is generally an ascending process, it was considered to be unrelated to FB_1 exposure. The occasionally observed hydronephrosis was considered background pathology.

No statistically significantly differences were observed between groups with respect to the activities of the kidney function parameters, urea and creatinine, measured in the serum (Table 5). Sphinganine and sphingosine levels as well as ratios were statistically significantly increased in the kidney tissues of all treatment groups (Table 6).

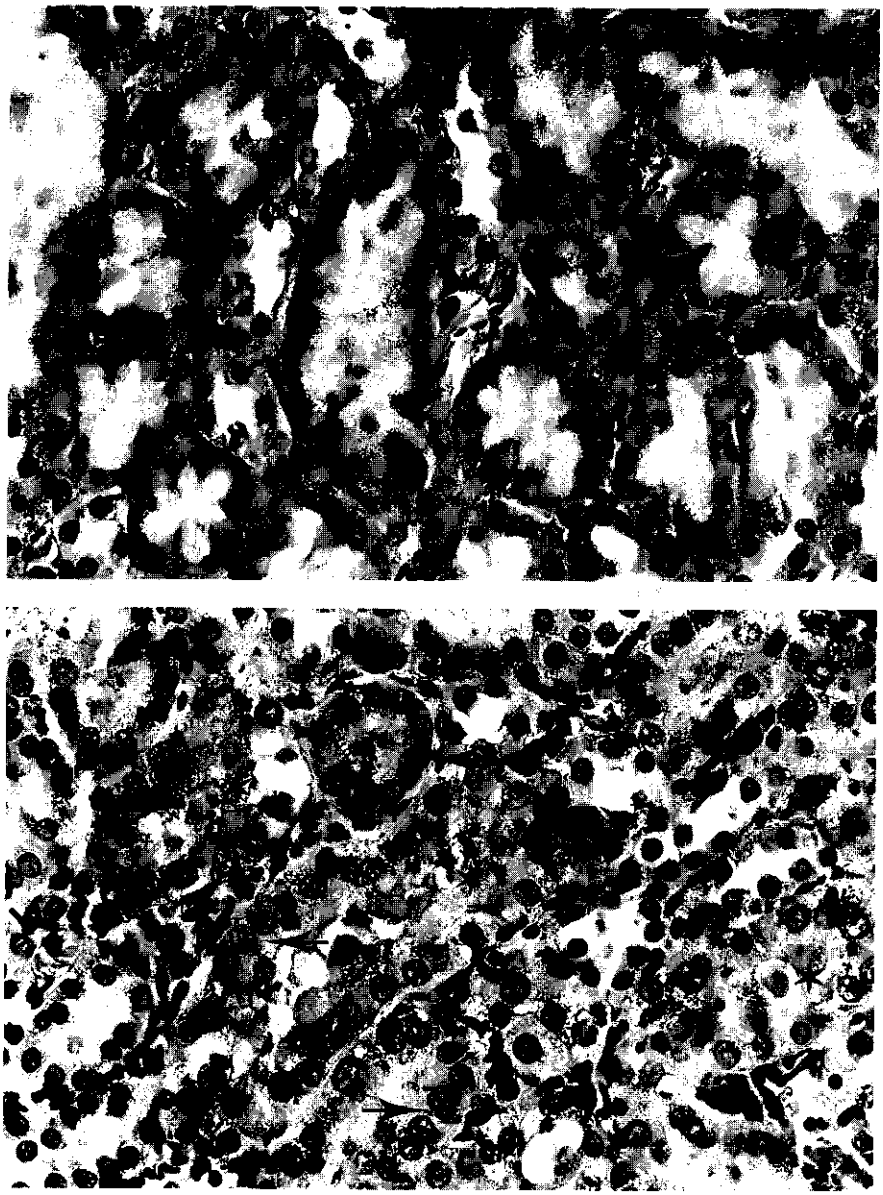

Figure 1. *Kidneys of rats from the control and high dose group. Top: Kidney from animal belonging to the control group, showing outer stripe of outer medulla (x 440). Bottom: Kidney from animal belonging to the high dose group, treated with fumonisin B_1 (x 440). Note presence of death cells in lumen of tubules (asterisks), increased density of enlarged nuclei (arrows), and mitosis (arrowhead).*

Table 3. *Effect of fumonisin B_1 treatment on haematological parameters in rats (Mean ± SD, n=10 per group).*

	Control	Low dose	Mid dose	High dose	P*
number of animals (except femural bone marrow) ‡	8	9	8	10	
WBC ($x10^9$/l)	9.32±2.29	8.91±1.89	9.56±1.07	8.43±1.26	0.5133
RBC ($x10^{12}$/l)	7.65±0.53	7.65±0.43	7.65±0.33	7.47±0.39	0.7371
HGB (mM)	9.04±0.48	9.19±0.35	9.14±0.34	8.86±0.44	0.3235
HCT (l/l)	0.43±0.03	0.43±0.02	0.43±0.01	0.42±0.02	0.6990
MCV (fl)	56.49±2.41	56.90±1.4	56.50±1.4	56.80±0.9	0.9273
MCH (fmol)	1.18±0.06	1.20±0.03	1.20±0.03	1.19±0.02	0.7712
MCHC (mM)	20.97±0.35	21.16±0.19	21.16±0.31	20.86±0.28	0.0854
RDW (%)	13.4 ±1	13.2 ±0.5	13.2 ±0.6	13.4 ±0.6	0.8725
HDW (%)	1.20±0.03	1.21±0.04	1.21±0.08	1.20±0.05	0.9027
PLT ($x10^9$/l)	1096±107	1046±103	1006±152	1112±86	0.2071
MPV(fl)	5.5 ±0.2	5.4 ±0.2	5.3 ±0.2	5.4 ±0.3	0.3198
Neut abs ($x10^9$/l)	0.66±0.27	0.63±0.18	0.84±0.56	0.70±0.12	0.5443
Lymph abs ($x10^9$/l)	8.31±2.22	7.96±1.76	8.25±1.00	7.39±1.23	0.6107
Mono abs ($x10^9$/l)	0.17±0.06	0.15±0.04	0.19±0.09	0.17±0.04	0.6416
Eos abs ($x10^9$/l)	0.06±0.03	0.07±0.02	0.16±0.20	0.10±0.09	0.2673
Baso abs ($x10^9$/l)	0.02±0.01	0.02±0.01	0.02±0	0.02±0.01	0.3780
Luc abs ($x10^9$/l)	0.09±0.03	0.08±0.03	0.10±0.03	0.07±0.02	0.1895
Neut (%)	7.44±3.48	7.18±1.88	8.69±5.23	8.50±2.45	0.7369
Lymph (%)	8.81±4.05	89.13±2.15	86.46±5.76	87.37±2.77	0.4502
Mono (%)	1.88±0.52	1.73±0.36	1.96±0.79	2.00±0.50	0.7382
Eos (%)	0.67±0.21	0.8 ±0.3	1.71±2.10	1.12±0.95	0.2793
Baso (%)	0.2 ±0.07	0.22±0.06	0.19±0.05	0.19±0.04	0.5149
Luc (%)	0.98±0.14	0.93±0.15	0.96±0.21	0.82±0.21	0.2189
Femural Bone marrow ($x10^9$/l)	26.05±5.07	28.35±5.91	24.58±6.44	25.36±3.15	0.5069
animals per group	10	10	10	10	

* P-value of 1-factor ANOVA

‡ The blood of animals 1, 10, 17, 21 and 23 was not analysed due to presence of blood clots

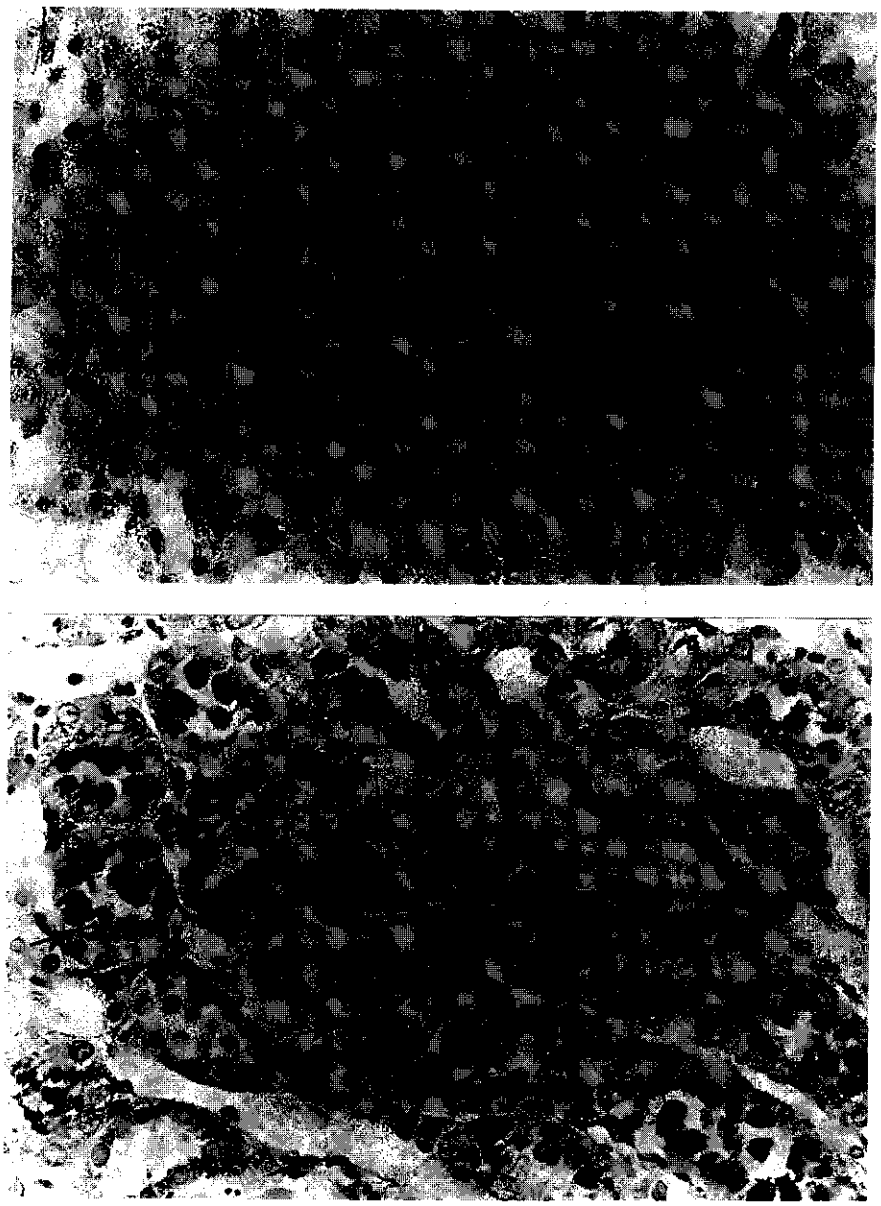

Figure 2. *Immunohistochemical staining of kidneys of rats from the control and high dose group. Top: Kidney of animal belonging to the control group, showing an occasionally apoptotic cell (arrow) in the outer stripe of the outer medulla (440x). Bottom: Kidney of animal belonging to the high dose group, treated with fumonisin B_1 (440x). Note the many apoptotic cells (arrow) in the lumina of the tubules.*

Table 4. *Incidence of kidney lesions in rats after oral administration of fumonisin B_1.*

Group:	Control	Low dose	Mid dose	High dose
Sex:	Male	Male	Male	Male
mg fumonisin B_1 kg^{-1} BW:	0.00	0.19	0.75	3.00
Number examined	10	10	10	10
Not remarkable	1	0	0	0
Tubular dilatation	1	1	1	1
minimal	1	1	1	1
Hydronephrosis	1	2	0	0
minimal	0	1	0	0
slight	0	1	0	0
moderate	1	0	0	0
Tubular cell death	0	5	10	10
minimal	0	3	0	0
slight	0	2	4	0
moderate	0	0	4	5
marked	0	0	1	5
severe	0	0	1	0
Basophilic tubules	9	10	10	10
minimal	8	8	0	0
light	1	1	0	0
moderate	0	0	1	0
marked	0	1	1	0
severe	0	0	8	10
Interstitial nephritis	0	0	5	2
minimal	0	0	4	2
slight	0	0	1	0
Pyelitis	0	0	2	0
minimal	0	0	1	0
moderate	0	0	1	0

Histologically, no indications for liver toxicity were found. However, ALAT and ASAT activities, indicative for liver function, were statistically significantly decreased in serum of animals in all treatment groups and the low dose group, respectively (Table 5). The decrease of activity in both assays was marginal and not dose related and was, therefore, considered not significant to FB_1 toxicity. γ-GT activity measured

in serum was slightly but statistically significantly increased in animals of the mid and high dose groups (Table 5). The level of sphinganine in liver tissue of animals of all treatment groups was statistically significantly increased (Table 6). Sphingosine level was slightly increased (not dose related) but sphinganine-to-sphingosine ratio showed a dose related increase, though not statistically significant, in liver tissue.

Table 5. *Effect of fumonisin B_1 treatment on liver and kidney functions in rats (Mean ± SD).*

		Control	Low dose	Mid dose	High dose	P*
Kidney	Urea (mM)	8.3±1.4	8.6±1.0	8.7±1.9	8.7±1.5	0.9210
	Creat (µM)	50.3±5.7	49.9±3.9	50.0±7.5	46.5±6.2	0.4539
Liver	ALAT (U/l)	64±27	43±10[a]	42±6[a]	44±13[a]	0.0109
	ASAT (U/l)	88±29	66±10[a]	68±9	71±12	0.0316
	γ-GT (U/l)	0.064±0.1	0.012±0.04	0.287±0.23[a]	0.478±0.33[a]	3.9×10^{-5}

* P-value of 1-factor ANOVA

[a] Statistically significantly different from control group (P<0.05)

Macroscopically, haemorrhages in mandibular lymph nodes (17 animals), thymus (2 animals) or popliteal lymph nodes (2 animals) were observed incidentally in all treatment groups, and considered to belong to background pathology.

No statistically significantly differences, due to FB_1 treatment, were observed in immunoglobulin concentrations in serum (Table 7), mitogen responsiveness of lymphoid spleen cells (Table 8) and natural killer cell activity between the groups (Table 9) for animals belonging to the various groups.

Results of the analysis of subpopulations of splenic lymphocytes are shown in Table 10. Non-dose related increases were observed in T-helper cell ($CD4^+$-cell, ER-2) and T-suppressor cell ($CD8^+$-cell, OX-8) subpopulations. The B-cell populations (%) were increased (non-dose related) in all treatment groups, with statistically significantly increases in the low and high dose groups. An increase in total T-cell population ($CD3^+$-cell, OX-19) (%) was observed in all treatment groups, with statistically significantly increases in animals in the mid dose group. No statistically

significantly changes were observed when the data were expressed as absolute cell numbers per spleen.

Treatment with FB_1 had no effects on sphingolipid concentrations in the serum of the animals (Table 7).

Table 6. *Effects of fumonisin B_1 treatment on sphingolipid levels in serum and tissues of kidney and liver in rats (Mean±SD). Number in brackets is number of animals if not 10.*

	Control	Low dose	Mid dose	High dose	P*
Sphinganine					
Serum (nM)	77.6±46	57.7±19	73.3±50	45.6±21	0.2064
Kidney (pmol/mg)	1.5±0.6	80.9±51.3[a]	125.8±67.4[a]	297.7±166.7[b]	$3x10^{-7}$
Liver (pmol/mg)	0.5±0.2	0.7±0.3 (9)	1.3±1.0[a]	1.5±1.3[a]	0.0484
Sphingosine					
Serum (nM)	189.9±73	188.4±77	184.6±77	206.6±256	0.9868
Kidney (pmol/mg)	8.4±2.7	33.9±13.0[a]	28.6±11.5[a]	56.1±22.1[b]	$5x10^{-8}$
Liver (pmol/mg)	6.4±2.4	13.1±11.6 (9)	12.6±8.2	9.4±6.5	0.2213
Ratio sphinganine-to-sphingosine					
Serum	0.45±0.23	0.35±0.17	0.39±0.13	0.35±0.17	0.5707
Kidney	0.18±0.07	2.56±1.44[a]	4.33±1.35[b]	5.25±1.39[b]	$1x10^{-10}$
Liver	0.09±0.03	0.08±0.04 (9)	0.11±0.07	0.26±0.34	0.0811

* P-value of 1-factor ANOVA

[a] Statistically significantly different from control group ($P<0.05$)

[b] Statistically significantly different from control and low dose groups ($P<0.05$)

DISCUSSION

This 28-day study on toxicity of low doses of FB_1 in rats, including immune function tests, revealed the kidney as the prime target organ for toxicity in this animal species. A dose-response relationship was observed for presence of apoptotic cells in kidneys of animals of all treatment groups.

Table 7. *Effects of fumonisin B_1 treatment on immunoglobulin levels (%) in rats (Mean ± SD). The number of animals per group is given in brackets if not 10.*

	Control	Low dose	Mid dose	High dose	P*
IgM (%)	107±26	115±25	107±26	107±22	0.8723
IgG (%)	105±44	74±21	95±22	97±21	0.1241
IgA (%)	101±59	127±44	137±60	133±77	0.5439
IgE (%)	98±80	73±76	82±55	52±35	0.4562

* P-value of 1-factor ANOVA

Table 8. *Effects of fumonisin B_1 on mitogen responsiveness of lymphoid spleen cells in rats presented as cpm (counts per minute) per culture (Mean±SD, n=10 per group). Blanks (=medium) are subtracted.*

	Control	Low dose	Mid dose	High dose	P*
Unstimulated	1836±333	1703±533	1697±444	1623±474	0.7661
ConA	120196±18738	115768±16986	116935±11610	110218±20546	0.6340
PHA	96064±25973	98433±27053	100437±25237	93043±32724	0.9415
LPS	5304±1952	5004±2564	5091±1949	5451±2393	0.9691

* P-value of 1-factor ANOVA

Table 9. *Results of natural killer cell activity of rats exposed to fumonisin B_1 expressed as % cytotoxicity release (Mean±SD, n=10 per group).*

Effect:target cell ratio	Control	Low dose	Mid dose	High dose	P*
200:1	67±10†	65±11	68±11	65±15	0.8837
100:1	52±11	49±17	53±16	49±17	0.8776
50:1	30±10	31±15	35±19	31±14	0.9042
25:1	16±5	15±9	18±11	19±7	0.7476

* P-value of 1-factor ANOVA

† Control group in 200:1 statistically significantly difference between animals (5) sacrificed on day 28 and the animals (5) on day 29

FB_1 treatment did not affect body weight, at the dosage levels used. Kidney weights of animals in the high dose group were statistically significantly reduced. On the contrary, FB_1 administrated at $\geq$ 450 mg FB_1 kg^{-1} feed to broiler chicks resulted in increased kidney weights *(31)*. Extensive single cell death was observed in the medulla of the kidneys of the mid and high dose treatment groups in this experiment. The majority of nephrotoxic substances affect proximal renal tubules, which are particularly sensitive to toxic substances due to their high energy demand (reabsorbative and secretory functions) *(18)*. Single cell death in the tubuli of the kidney in rats following exposure to FB_1 has been observed in several other studies *(2, 25, 27, 28, 29)*. Basophilic tubules were indicative for the ongoing regeneration process in the mid and high dose groups in this experiment. Induced mitosis was observed when rats were exposed to 0.75 and 3.0 mg FB_1 kg^{-1} BW per day ($\cong$ 7.5 and 30 mg kg^{-1} feed, respectively). Mitosis in the kidneys of rats with necrotic cells present in the kidneys was also observed in a 90-day subchronic feeding study in rats *(27)*. The presence of proliferating cells and lack of a severe inflammatory reaction indicates that the FB_1 induced cell death in kidneys of rats might result from apoptosis. This was confirmed by specific immunohistochemical staining. Cell proliferation and differentiation were not sufficient to compensate the loss of kidney weight, caused by cell death, in animals in the high dose group in this study. Identical findings in rats were reported by Voss *et al.* (1995a) at a level of 9 mg FB_1 kg^{-1} feed per day (= 0.7 mg FB_1 kg^{-1} BW on average for female and male rats). Increased rate of cell proliferation can cause insufficient differentiation of the cells in the tubules of the kidneys in rats which may induce tumorous alterations in the proliferating cells or may severely damage the kidney functions.

The results of the measured biochemical parameters for liver function, show liver toxicity at a daily dose of 3.0 mg FB_1 kg^{-1} BW in rats ($\cong$ 30 mg FB_1 kg^{-1} feed). A dose 16 times higher than the dose for kidney toxicity. Primary hepatocellular carcinoma were detected in rats after daily administration of 3.75 mg FB_1 kg^{-1} BW (= 50 mg FB_1 kg^{-1} feed) for 26 months *(8)*, a level slightly above the one at which liver toxicity was observed in this study. An effective dosage level (EDL) of between 7 and 15 mg FB_1 kg^{-1} BW per day was found for cancer initiation in rat liver over a 21-day period *(6)*.

Cancer promotion activity in rat liver was observed when feeding a diet containing 50 mg FB_1 kg^{-1} feed for 21 days to diethylnitrosamine-initiated rats *(9)*.

Table 10. *Results of splenic lymphocyte subpopulations of rats exposed to fumonisin B_1 (Mean ± SD, n=10 per group).*

Cells		Antibodies	Control	Low dose	Mid dose	High dose	P*
			Percentage (%):				
B-cells		MARK-1	34.0±3.5	38.0±4.4[a]	35.4±1.9	38.2±2.7[b]	0.0145
Total T-cells	CD3+	OX-19	42.5±15.3	52.8±4.8	53.5±3.9[a]	50.7±3.2	0.0217
T-helper cells	CD4+	ER-2	37.0±13.2	40.6±2.4	41.1±2.4	42.5±3.6	0.3605
T-suppressor cells	CD8+	OX-8	25.5±7.1	30.5±3.7	29.2±2.8	27.7±1.5	0.0765
Total number of cells per spleen ($x10^7$)			45.4±9	48.2±9	47±6	44±9	0.7211
			Number of cells per spleen ($x10^7$) for each subpopulation:				
B-cells		MARK-1	15.3±2.7	18.3±4.1	16.7±2.6	16.7±3.4	0.2394
Total T-cells	CD3+	OX-19	19.6±9.2	25.6±6.1	25.1±3.2	22.4±5.1	0.1367
T-helper cells	CD4+	ER-2	17.1±8.2	19.6±4.0	19.2±2.1	18.6±3.2	0.6844
T-suppressor cells	CD8+	OX-8	11.7±4.6	14.8±3.9	13.7±2.1	12.2±2.8	0.1949

* P- value of 1-factor ANOVA

[a] Statistically significantly different from control group (P<0.05)

[b] Statistically significantly different from control and mid dose groups (P<0.05)

B-cell and T-cell populations (%), subpopulations of splenic lymphocytes, were statistically significantly increased in several treatment groups as opposed to animals in the control group in this study. Reduced thymus weight, disseminated thymic necrosis and consistently elevated serum IgM levels were observed after ip dosing of rats at 7.5 mg FB_1 kg^{-1} BW (≅ 75 mg kg^{-1} feed) on a daily basis for 4 consecutive days *(2)*. Both stimulatory and suppressive effects on plaque forming cells against sheep red blood cells were observed in mice were dosed daily by ip

with 1 to 5 µg FB_1 per animal for 5 days *(15)*. Reduced splenic and thymic weight, reduced total immunoglobulin and IgG levels and reduced macrophage phagocytic activity (by 34 %) in chickens receiving feed amended with *F. proliferatum* culture material containing 61 mg FB_1 kg^{-1} for 6 weeks were reported *(19)*. Statistically significantly reduced lymphocyte blastogenesis was observed in calves receiving a diet that was naturally contaminated with 148 mg FB_1 kg^{-1} feed for 31 days *(17)*. No statistically significantly effects on cell surface antigens (thymus, blood and spleen) nor on mitogen responsiveness of lymphoid spleen cells (PWM, ConA and PHA) were found in sows fed a diet containing 100 mg FB_1 kg^{-1} feed *(1)*. The above mentioned results indicate that the immune system might be a target for FB_1 toxicity at doses higher than those at which kidney and liver toxicity were observed. Pigs seem to be less sensitive than cattle, rats, mice or chickens in this respect. Further studies, including infection models, are required to confirm these findings.

Exposure to fumonisin B_1 in a 28-day toxicity study caused dose-related apoptosis in the kidneys of rats dosed daily at 0.19, 0.75 or 3.0 mg fumonisin B_1 kg^{-1} body weight by gavage. Chronic exposure may lead to serious harmful effects in this organ, including elevated chance on tumour formation. Seriously affected liver and immune system functions after chronic exposure to fumonisin B_1 can not be ruled out based on the toxic effects observed in this study.

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9

Risk assessment
- a general discussion

INTRODUCTION

The probability and severity of adverse health effects to the consumer following consumption of food containing a certain hazardous agent is estimated in this risk assessment. The risk assessment comprises the hazard identification, exposure assessment, hazard characterisation and the risk characterisation into an estimation of the occurrence and severity of known or potential health effects likely to occur in a given population including attendant uncertainties.

This risk assessment starts with discussing the results of the hazard identification *(Chapter 2)*, exposure assessment *(Chapters 3 to 7)* and hazard characterisation *(Chapter 8)*. The risk characterisation is described later in this chapter and emphasis will be put on fumonisin B_1 possibly present in food in The Netherlands.

RISK ASSESSMENT

Hazard identification

Twelve secondary metabolites, out of 137, produced by species of the genus *Fusarium* isolated from food raw materials, were characterised as toxic to animals in

toxicity studies *(Chapter 2)*. These mycotoxins can occur in food raw materials, knowing that the secondary metabolite production by the fungus also takes place in the field. Six of these twelve mycotoxins have been related to human intoxications; T-2 toxin (food poisoning and human casualties), nivalenol (food poisoning), deoxynivalenol (food poisoning and immunotoxic), acetyldeoxynivalenol (food poisoning), fumonisin B_1 (carcinogenic and related to human oesophageal cancer) and zearalenone (oestrogen). The other six mycotoxins belonging to the group of 12 are accordingly regarded as potentially toxic to human health.

Several of the secondary metabolites are produced concurrently by the fungus. The simultaneously produced secondary metabolites may alter their respective toxic effects. Basal cell hyperplasia was observed in the oesophagus of rats following exposure to *Fusarium moniliforme* culture material with unknown fumonisin B_1 (FB_1) contamination level *(9, 13)*. These effects have not been repeated when feeding a diet amended with purified FB_1 which suggests that toxicity of FB_1 is altered considerably by concurrently produced `minor metabolites`. Co-occurring mycotoxins in diets produced by other fungal species may alter toxic effects. Currently used analytical methods allow the detection of a limited number of mycotoxins and co-occurring secondary metabolites in food raw materials or food commodities. The concentration of the mycotoxin can, therefore, be regarded as indicative for the toxicological quality of the commodity investigated.

Exposure assessment

Prevalence of *Fusarium* infection and occurring species in cereals and thus in potential mycotoxin contamination varied considerable between sampling years 1991 and 1993, as a result of environmental conditions. Eight *Fusarium* species were isolated from cereals grown in The Netherlands *(Chapter 3)*. A substantial genotypic and phenotypic variation was observed within two of the most frequently isolated *Fusarium* species *(Chapter 4)*. The eight *Fusarium* species that were isolated can potentially produce at least 16 secondary metabolites of which 6 were identified as mycotoxins in the hazard identification *(Chapter 2)*. These potentially produced mycotoxins can occur in cereals grown in The Netherlands.

Table 1. *Daily intake of maize by populations in various regions of the world and by people belonging to the group at risk. Mean value in g per person, per day.*

Country, region or group of people	Mean daily maize intake (g) per person		Year of survey or publication[#]	Group of survey	
	All people	'Eaters only'			
Countries					
The Netherlands (maize)	3.0	*42*[*‡]	1992	Population	*(3)*
The Netherlands (canned maize)	1.4	*19*[*‡]	1992	Population	*(3)*
European Union	*7.2*[§]	*99*[‡]	1994[#]	Population	*(20)*
Canada	2.6	36	1970-1972	Adults	*(12)*
Transkei	460.0	-	1988[#]	Adults	*(21)*
Groups at risk					
People with celiac or Dühring's disease[$]	*162*	-	1992	Population	*(3)*
FAO/WHO Global diets					
Global diet	56.8	-	1994[#]	Adults	*(1)*
Middle East	48.3	-	1994[#]	Adults	*(1)*
Far East	31.2	-	1994[#]	Adults	*(1)*
Africa	106.2	-	1994[#]	Adults	*(1)*
Latin America	41.8	-	1994[#]	Adults	*(1)*

[#] Year of publication

[*] Data printed in italics are calculated by the author, based on assumptions.

[‡] Calculated, assuming ratio all eaters to 'eaters only' as in Canadian survey.

[§] Calculated from wheat consumption.

[-] All people in survey are considered eaters.

[$] Based on diet in The Netherlands

Two wheat samples (n=69) of batches harvested the 1993 in The Netherlands were contaminated with deoxynivalenol (DON) at levels of 500 (limit of determination) and 750 ng DON g^{-1} uncleaned grain, respectively. One barley sample was contaminated with 270 ng zearalenone (ZEA) g^{-1} uncleaned grains (limit of determination 200 ng ZEA g^{-1} grain) *(Chapter 3)*.

FB_1 contamination is mainly restricted to maize. All maize for human consumption is imported in The Netherlands since climatic conditions do not allow the ripening of maize. FB_1 was detected in 98 % of maize imported in The Netherlands and intended for the production of food *(Chapter 5)*. Low incidence and contamination levels of FB_1 were found in maize-containing food commodities, regardless of the assumed stability of the mycotoxin to process conditions *(Chapter 6)*. Mycotoxins may react with food compounds during food processing or the used analytical procedure may fail to extract the mycotoxins from the matrix.

Inventory and monitoring studies on mycotoxin contamination of food raw materials and relevant food commodities allow accurate estimation of human exposure and may in addition give an indication of the severeness of the adverse effects to human health. The median value of contamination is advised for calculating the mycotoxin intake when a limited number of data on mycotoxin contamination is available. This will avoid calculation problems when the concentration of the mycotoxin in a sample is below the limit of determination, as opposed to the use of the mean value *(10)*. Using the maximum contamination level will, in general, overestimate the risks.

The accurate estimation of human exposure to a certain mycotoxin requires data on the intake of the relevant food commodities and data on mycotoxin contamination of those commodities. Data on the intake of the separate food ingredients, such as maize, will suffice when the mycotoxin is stable to food processing conditions, such as FB_1. Table 1 (partly presented in *Chapter 7)* shows the maize intake by populations in various countries and regions of the world. The average intake of maize varies from 3 g per person per day in The Netherlands to 460 g per person in Transkei, with a global average intake of 56.8 g maize per person per day. Within each population or group the maize intake is distributed around the average.

Groups in the population at risk for high mycotoxin intake, in case of FB_1 the people with a high intake of maize, should be identified and considered separately in the risk characterisation. The group of `eaters only` in Table 1, is defined in the Canadian study as: people in the study that indicated the consumption of maize. People in this group consume 14 times more maize than the average amount and, probably, might enhance the average maize consumption of the total population. People with an intolerance to gluten (people with celiac or Dühring's disease) replace wheat, barley, rye and oats in their diet with maize and rice.

Fig. 5 in *Chapter 7* shows the probability of daily exposure of the population in The Netherlands to a certain average amount of FB_1. Data are calculated from maize intake and FB_1 contamination levels in maize.

Hazard characterisation

Fusarium mycotoxins are world-wide recognised as hazards to the health of the population and regulations are recommended for several of the mycotoxins, as is shown in Table 2 *(4)*. Regulatory limits should regard data on toxicity and mycotoxin contamination of the commodities. Table 2 shows that for a wide variety of commodities highly variable limits are prescribed. The DON levels in the two cereal samples harvested in The Netherlands in 1993, as described in *Chapter 3*, were above the recommended limits in Austria for wheat and rye and were below the recommended limits in Russia, USA and Canada. All DON levels were within the recommended limits for feed. One cereal sample was contaminated with ZEA above the recommended limit in Romania and Austria for food, wheat and rye and below the recommended limits in Brazil, France, Uruguay and Russia. Eighteen percent of the maize samples imported in The Netherlands and intended for food production was contaminated with FB_1 in levels above 1,000 ng FB_1 g^{-1} maize *(Chapter 5)*. One of the 78 samples from maize-containing food commodities was contaminated with FB_1 at a level over 1,000 ng FB_1 g^{-1} commodity *(Chapter 6)*. In comparison, the proposed limit in Switzerland is 1,000 ng g^{-1} maize for the sum of fumonisins B_1 and B_2. As a consequence, a large part of the batches maize imported in The Netherlands would be rejected for food preparation purposes.

Table 2. *World-wide recommended regulatory limits on* Fusarium *mycotoxins (4).*

Mycotoxin	Country	Commodity	Limit ng g^{-1}
Food			
All mycotoxins	Netherlands	Cereal(product)s, pulse(products)s, legume(product)s	0
T-2 toxin	Russia	Cereals (wheat of hard and strong types), flour, wheat bran	100
Deoxynivalenol	Austria	Wheat, rye	500
	Austria	Durum wheat	750
	Russia	Cereals (wheat of hard and strong types), flour, wheat bran	1,000
	USA	Finished wheat products	1,000
	Canada	Uncleaned soft wheat	2,000
Fumonisins $B_1 + B_2$	Switzerland	Maize(products)	1,000
Zearalenone	Romania	All foods	30
	Austria	Wheat, rye	60
	Austria	Durum wheat	60
	Brazil	Maize	200
	France	Cereals, vegetable oils	200
	Uruguay	Maize, barley	200
	Russia	Cereals (wheat of hard and strong types), flour, wheat bran	1,000
	Russia	Leguminous, protein isolators and concentrators, vegetable oil	1,000
	Russia	Nuts (kernel)	1,000
Feed			
All mycotoxins	Canada	Feedstuffs for reproducing animals	0
T-2 toxin	Israel	Grain for feed	100
HT-2 toxin	Canada	Diets for swine/young calves/lactating dairy animals	25
	Canada	Diets for cattle/poultry	100
Deoxynivalenol	Romania	All feedstuffs	5
	Canada	Diets for swine/young calves/lactating dairy animals	1,000
	Canada	Diets for cattle/poultry	5,000
	USA	Grains and grain by-products (not exceeding 40 % of the diet)	5,000
	USA	Grains and grain by-products destined for swine (not exceeding 20 % of the diet)	5,000
	USA	Grains and grain by-products destined for ruminating beef and feedlot cattle older than 4 months and for chickens (not exceeding 50 % of the cattle or chicken total diet)	10,000

To contribute data for the establishment of regulatory limits, a dose-response study was carried out with FB_1 in rats in a 28-day experiment *(Chapter 8)*. Aim was to investigate the toxicological profile of low doses of FB_1 with emphasis put on immunotoxic effects. The results of the dose-response toxicity study will be used to estimate a tolerable daily intake (TDI). The guidelines for estimating the acceptable daily intake (ADI) for food additives and contaminants, as published by the Joint FAO/WHO expert committee on Food Additives (JECFA), will be followed to estimate the TDI for FB_1 *(8)*. The expression `tolerable` will be used instead of `acceptable` to account for the natural occurrence of mycotoxins as opposed to the food additives. The procedure for estimating the TDI is identical to the procedure for estimating the ADI. Formally, the JECFA guidelines on food additives and contaminants may not be used to estimate a TDI for mycotoxins since mycotoxins are no food additives and have no necessary technological purpose. JECFA provides guidelines to calculate a provisional maximum tolerable daily intake (PMTDI) for food contaminants that do not accumulate in the body but specific guidelines on mycotoxins are not yet available.

The ADI for a food additive that does not accumulate in the body is defined by the JECFA as `an estimate by JECFA of the amount of a food additive, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk` *(8)*. A safety factor of 100 is implemented for food additives to estimate the ADI from the no observed effect level (NOEL). The safety factor provides an adequate margin of safety for the consumer by assuming that the human being is 10 times more sensitive than the test animal and that the difference of sensitivity within the human population is in a 10-fold range *(8)*. The NOEL for food additives is defined by JECFA as `the greatest concentration or amount of an agent, found by study or observation, that causes no detectable, usually adverse, alteration of morphology, functional capacity, growth, development, or lifespan of the target` *(8)*. There is a tendency to replace the NOEL and the lowest observed effect level (LOEL) by the no and lowest observed adverse effect level (NOAEL and LOAEL), however, expert judgement is currently not available to define the use of this expression. A NOEL should be established in at least two animal species and the

lowest NOEL, established in the most sensitive species, will be used to estimate the ADI. The threshold approach is justified (estimation of NOEL) in case of FB_1 because of the current knowledge on kidney toxicity and the negative genotoxic results *(6, 7, 14, 15)*. If FB_1 proves to be an ultimate carcinogen in long-term studies, as currently undertaken at the USDA (Jefferson, Arkansas, USA), no threshold level can be estimated.

A provisional tolerable weekly intake (PTWI), derived from the LOAEL, should be estimated for substances that accumulate in the body, such as the mycotoxin ochratoxin A. The PTWI for this mycotoxin was estimated by JECFA at 100 ng ochratoxin A kg^{-1} human BW (safety factor of 500) *(11)*.

FB_1 is water soluble and is poorly absorbed from the rat gut after gavage and rapidly eliminated from the body by bilary excretion *(17, 18)*. When radioactive labelled FB_1 was administrated to vervet monkeys by gavage, 64 % of the radioactivity was recovered after 24 hours in the excreta *(19)*. Administration of radioactive labelled FB_1 to swine by gavage showed low bioavailability of the mycotoxin, 3-6 %, and a rapid elimination of the mycotoxin from the plasma *(16)*. FB_1 does not accumulate in the bodies of test animals. It can, therefore, be assumed that the mycotoxin does not accumulate in the human body either. A TDI can, thus, be estimated, which will provide the means for a preliminary prediction based on currently known facts of possible risks to the public health due to FB_1 contamination of food in The Netherlands.

The horse is considered to be the most sensitive species for FB_1 toxicity, followed by monkey, rat, pig, hamster and mouse *(12)*. The rat is more sensitive to FB_1 than the mouse and male animals of these species are affected more severely than females under laboratory conditions *(23)*.

In *Chapter 8*, dose-response related toxic effects were observed in the kidneys of rats in all three treatment groups (0.19, 0.75 and 3 mg FB_1 kg^{-1} body weight (BW) per day): high incidence of apoptotic and proliferating cells in the kidneys and low kidney weight in animals of the high dose group, moderate incidence of apoptotic cells and presence of proliferating cells in kidneys of animals in the mid dose group and low incidence of apoptotic cells in kidneys of animals in the low dose group. The

LOEL for kidney toxicity in rats was, thus, 0.19 mg FB_1 kg^{-1} BW per day. A NOEL for kidney toxicity was not established in this study. However, a NOEL can be estimated by extrapolation, since the observed toxic effects were dose related. The FB_1 concentrations used in the dose-response toxicity study differed by a factor of four. The NOEL in rats might, therefore, be expected at a FB_1 concentration four times below the LOEL, at 0.05 mg FB_1 kg^{-1} BW. The TDI derived from this estimated NOEL would be 500 ng FB_1 kg^{-1} human BW (safety factor of 100).

A NOEL of 3 mg FB_1 kg^{-1} feed (= 0.23 mg FB_1 kg^{-1} BW per day, average for female and male animals) for kidney toxicity in rats was established in a subchronic dose-response feeding study *(23)*. A TDI of 2300 ng FB_1 kg^{-1} human BW can be derived from this NOEL (safety factor of 100).

The NOEL in horses was established at 0.1 mg FB_1 kg^{-1} BW based on an ELEM outbreak *(12)*. Toxic effects of co-occurring metabolites can not be excluded in this case and the JECFA guidelines can, officially, not be implemented. However, when using the NOEL for FB_1 in horses, the TDI can be estimated at 1000 ng FB_1 kg^{-1} human BW (safety factor of 100).

It should be taken into consideration that certain groups in the population, e.g. children, might have a higher sensitivity for FB_1 toxicity. Other issues of concern are the synergistic effects on toxicity caused by concurrently occurring secondary metabolites in food as discussed in the hazard identification. The results of the hazard characterisation showed that immunotoxic effects of FB_1 can not be ruled out. Co-occurring mycotoxins, such as DON with known immunotoxic characteristics, may significantly contribute to these effects, which may result in increased sensitivity of farm animals to zoonotic diseases and thus increase human exposure to zoonotic diseases.

Summarising, TDIs of 500 and 2300 ng FB_1 kg^{-1} human BW were derived from the NOELs estimated in toxicity studies in rats with purified FB_1 administered by gavage and amended food, respectively. A TDI of 1000 ng FB_1 kg^{-1} human BW was calculated from FB_1 related toxic effects observed in horses.

Risk characterisation

The risk characterisation is the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known potential adverse health effects in a given population *(2, 5)*.

The part of the population (%) exposed to a certain amount of FB_1 per day is estimated in this risk assessment. The estimation will be based on the estimated TDI in the hazard characterisation and the results on exposure assessment shown in Fig. 5 *Chapter 7*. The health consequences of excursion of the TDI will not be discussed here, since toxicological data are not available in this respect. However, the presence of basophilic tubules and mitotic cells found in the kidneys of rats in the hazard characterisation *(Chapter 8)*, may indicate a rapid regeneration of kidney tissue after withdrawal of FB_1 from the diet.

The hazard characterisation revealed a LOEL of 0.19 mg FB_1 kg^{-1} BW in rats. This is equal to an intake of $1x10^7$ ng FB_1 per person per day (body weight of 74 kg). Fig. 5 in *Chapter 7*, iso-line 7, shows that no person in the Netherlands population consuming the average amount of maize or belonging to the group of 'eaters-only' is exposed daily to this level. Less than one percent from the group of people with gluten intolerance is exposed daily to this level of FB_1.

The TDI for FB_1 was estimated at a level between 500 and 2300 ng FB_1 kg^{-1} human BW (described in the hazard characterisation in this chapter). The average body weight for people over 18 years of age in The Netherlands is 74 kg *(22)*. The corresponding tolerable daily intake per person (TDI_{pp}) can, roughly, be estimated at a level between $1x10^4$ and $1x10^5$ ng FB_1 in The Netherlands.

Considering the TDI_{pp} and Fig. 5 in *Chapter 7*, the probability of being exposed to FB_1 at a level corresponding to the TDI_{pp} of $1x10^4$ ng FB_1 (iso-line 4) would be 12 % for the people in The Netherlands consuming the average amount of maize per day, 55 % for people belonging to the group of `eaters only` and 78 % for people with gluten intolerance. In case of a TDI_{pp} of $1x10^5$ ng FB_1 (iso-line 5) the percentages exposed would be, 1 %, 16 % and 37 %, for the respective groups.

The use of the median or mean FB_1 contamination level will estimate the daily exposure of the whole population or the distinguished group as a whole, to a certain

average level of FB_1. This approach is illustrated in the study of Kuiper-Goodman and co-workers for the Canadian situation *(12)*. FB_1 contamination levels were low in maize grown in Canada and, therefore, exposure of the Canadian population to FB_1 present in Canadian maize was accordingly low. They concluded that negative effects on the public health due to FB_1 intake could be ruled out for all groups of the population in Canada.

A median and mean level of 420 and 1,359 ng FB_1 g^{-1} maize, respectively, were derived from the data on FB_1 contamination in maize in *Chapter 7*. Table 3 shows the daily FB_1 intake (ng FB_1 per person) by people of the various groups when using the median or mean FB_1 contamination levels of maize. No people in The Netherlands consuming the average amount of maize will be exposed to FB_1 at a level corresponding to the TDI_{pp} of $1x10^4$ or $1x10^5$ ng FB_1. All people in the group of `eaters only` are exposed to FB_1 at a level corresponding to the TDI_{pp} of $1x10^4$. In case of a TDI_{pp} of $1x10^5$, the people belonging to the group of `eaters only` will be exposed to this level when the mean is used to calculate FB_1 intake. All people with gluten intolerance are exposed to FB_1 levels corresponding to the TDI_{pp} of $1x10^4$ and $1x10^5$ ng FB_1, regardless of the calculation method.

Table 3*. Daily fumonisin B_1 intake (ng FB_1 per person) by various groups of the population calculated using median and mean value of contamination.*

	Consumption of maize per person per day g	Calculation method	
		Median ng FB_1 g^{-1} maize 420	Mean ng FB_1 g^{-1} maize 1,359
Population on average	3	$1,260 \cong 1x10^3$	$4,077 \cong 1x10^3$
`Eaters only`	42	$17,640 \cong 1x10^4$	$57,078 \cong 1x10^5$
People with gluten intolerance	162	$68,040 \cong 1x10^5$	$220,158 \cong 1x10^5$

It can be concluded, from the data presented in this study, that negative effects on the public health due to FB_1 intake can not be ruled out for certain groups in the population in The Netherlands. High FB_1 contamination levels of maize combined with high maize intake are the cause of high FB_1 exposure.

DIRECTIVES FOR RISK MANAGEMENT

The results described in this thesis clearly demonstrate that more research is needed in the fields of toxicity and exposure before risk management can be completed. Risk management, following the risk assessment, is required to weigh policy alternatives in the light of the results of the risk assessment and, if required, selecting and implementing appropriate control options. If an assessed risk is not acceptable, criteria (standards) for the tolerable presence of the mycotoxin should be set.

As mentioned, monitoring FB_1 contamination in food and investigating trends in maize consumption will enable a more accurate exposure assessment. The enumeration of the groups at risk and of `eaters only` should be established for the situation in The Netherlands along with data on maize intake by people belonging to these groups.

Chronic feeding studies on FB_1 would reveal data for a reliable estimation of the safety factors to establish a TDI. Data on toxic effects in case of excursion of the TDI should be generated. Human epidemiological studies on incidence of oesophageal cancer and kidney diseases would increase knowledge on the sensitivity of humans to the toxic effects of FB_1. These studies should focus on the groups at risk identified in this study.

The results on FB_1 toxicity and contamination of maize may be used to estimate a regulatory level for FB_1 in food.

The *Fusarium* mycotoxins DON (immunotoxic) and ZEA (oestrogen) were detected in cereals grown in The Netherlands and might be present in cereals imported in the country and in the food made from these cereals. This study generated evidence to propose risk assessment studies on the impact of DON and ZEA in food on the public health in The Netherlands.

Fusarium mycotoxins may alter the immune system of farm animals. The effects on incidence of infectious diseases and possibly increased human exposure to zoonotic infections should, therefore, be investigated.

CONCLUSION

The risk assessment shows that it cannot be excluded that the health of the population in The Netherlands in the current situation might be challenged by *Fusarium* mycotoxins present in food.

People in The Netherlands are currently not exposed to fumonisin B_1 at levels that caused kidney toxicity in rats. However, people of the population consuming maize regularly, the group of 'eaters only', and people with gluten intolerance might be exposed to levels above the estimated tolerable daily intake (TDI, based on the available data on toxicity in test animals and including safety factors). Kidney related problems can not be ruled out in the groups at risk.

Deoxynivalenol can be present in all cereals, including silage maize, grown or imported in The Netherlands. This mycotoxin can adversely affect the immune system of both humans and farm animals and, thus, possibly cause an increased exposure of the population to zoonotic diseases.

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SUMMARY

This study concerns the risk assessment of toxic secondary metabolites, mycotoxins, produced by species of the fungal genus *Fusarium*. The purpose of this study was to estimate the toxic effects of *Fusarium* mycotoxins present in food on the health of the population in The Netherlands by using a formal risk assessment approach.

The plant pathogenic *Fusarium* moulds occur world-wide and crops become infected during the growing period. Mycotoxin production may take place in the field, and mycotoxins can, therefore, occur in food raw materials, such as cereals. Most of the mycotoxins are stable under process conditions used for the production of food and feed. Carry-over of *Fusarium* mycotoxins from contaminated feed through animals on humans is currently not known. Species of the genus *Fusarium* can produce numerous secondary metabolites, of which a limited number has known toxic effects on human and animal health.

A literature review revealed twelve secondary metabolites, out of 137 produced by species of the genus *Fusarium* isolated from food raw materials, as toxic to animals in toxicity studies *(Chapter 2)*. Six of these twelve mycotoxins have been related to human intoxications: T-2 toxin (food poisoning and human casualties), nivalenol (food poisoning), deoxynivalenol (food poisoning and immunotoxic), acetyldeoxynivalenol (food poisoning), fumonisin B_1 (carcinogenic and possibly related to human oesophageal cancer) and zearalenone (oestrogen). The other six mycotoxins belonging to the group of 12 are accordingly regarded as potentially toxic to human health.

Exposure assessment of the population in The Netherlands to *Fusarium* mycotoxins was established by analysing cereal samples for *Fusarium* infection and mycotoxin contamination and by estimating intake of relevant foods by the population. As a result of environmental conditions, the incidence of *Fusarium* infection and occurring species and, thus, in potential mycotoxin contamination varied considerably between sampling years 1991 (34 % of samples infected) and 1993 (83 % of samples infected) *(Chapter 3)*. A considerable genotypic and

phenotypic variation was observed within two of the most frequently isolated *Fusarium* species *(Chapter 4)*. A total of eight *Fusarium* species was isolated from the cereals, which could potentially produce at least 16 secondary metabolites of which 6 were identified as mycotoxins in the hazard identification study. It was concluded that all the potentially produced secondary metabolites may occur in cereals grown in The Netherlands.

Samples from the 1993 crop (n=69) of cereals grown in The Netherlands and intended for food or feed production were contaminated with deoxynivalenol (3 % of the samples) or zearalenone (1 % of the samples) above the limit of determination (500 ng deoxynivalenol g^{-1} and 200 ng zearalenone g^{-1}) *(Chapter 3)*.

Maize for human consumption is imported in The Netherlands since the climate does not allow ripening of the crop. Ninety-eight percent of samples from imported batches of maize intended for food production (n=62) was contaminated with fumonisin B_1 *(Chapter 5)*. Low incidence and contamination levels of fumonisin B_1 were found in maize-containing food commodities, despite the assumed stability of the mycotoxin to process conditions *(Chapter 6)*.

The chance that the population in The Netherlands is daily exposed to certain mean levels of fumonisin B_1 was estimated using the data on fumonisin B_1 contamination of maize and the daily intake of maize by the population *(Chapter 7)*. Average maize consumption by the population in The Netherlands was estimated at 3 g maize per person per day. People belonging to the group `eaters only` (extrapolated from a Canadian survey in which they were defined as the group of people reporting the consumption of maize) consumed 42 g per person per day on average. The maize intake by people with gluten intolerance (people with celiac or Dühring's disease), the group at risk for high fumonisin B_1 intake, was estimated at 162 g maize per person per day. No data were available on variation in maize intake within a group or variations over a period of time.

The hazard characterisation concerned a 28-day dose-response toxicity study on fumonisin B_1 in rats *(Chapter 8)*. The toxicological profile and target organs were established for low doses of fumonisin B_1. Emphasis was put on possible immunotoxic effects of fumonisin B_1. The animals were dosed daily by gavage with

0, 0.19, 0.75 or 3 mg fumonisin B_1 kg^{-1} body weight. A dose-response related effect was observed for apoptosis in the kidneys of rats. Serious harmful effects in this organ, including elevated chance on tumour formation can not be excluded in case of chronic exposure. The lowest observed effect level was 0.19 mg fumonisin B_1 kg^{-1} body weight for kidney toxicity. Liver toxicity was observed in the highest dose group. There was evidence for toxicity towards the immune system at higher concentrations. A tolerable daily intake (TDI) of 500 ng fumonisin B_1 kg^{-1} human body weight was derived from the results of the dose-response study.

The probability of being exposed to fumonisin B_1 at a level corresponding to the estimated TDI was assessed in the risk characterisation. The results show that 12 % of the people in The Netherlands consuming the average amount of maize per day, 55 % of the people belonging to the group of `eaters only` and 78 % of the people with gluten intolerance are daily exposed to fumonisin B_1 at the level corresponding to the estimated TDI *(Chapter 9)*.

The health of the consumers, especially people belonging to the groups at risk, in The Netherlands might in the current situation be challenged by *Fusarium* mycotoxins present in food. Deoxynivalenol, which has immunotoxic characteristics, can potentially be present in food and feed and might increase human exposure to infectious diseases, especially to those from zoonotic origin.

SAMENVATTING

De hier gepresenteerde studie betreft de *risk assessment* van toxische secundaire metabolieten, de mycotoxinen, die door schimmels van het genus *Fusarium* geproduceerd worden. Het doel van deze studie was om de toxische effecten van *Fusarium* mycotoxinen aanwezig in levensmiddelen met betrekking tot de volksgezondheid in te schatten.

Fusarium schimmels komen wereldwijd voor en zijn in staat de meeste landbouwgewassen te infecteren. Als gevolg van de infectie kunnen mycotoxinen worden gevormd. De mycotoxinen komen voor in grondstoffen, zoals granen, bestemd voor de productie van levensmiddelen. De meeste mycotoxinen zijn stabiel en worden nauwelijks beïnvloed door de processen die gebruikt worden bij de verwerking van de grondstoffen tot levensmiddelen. Carry-over van mycotoxinen uit gecontamineerd voer via dieren op mensen is op dit moment niet bekend voor de mycotoxinen van *Fusarium*. Species van het genus *Fusarium* kunnen een groot aantal verschillende mycotoxinen uitscheiden, maar slechts van een klein aantal hiervan zijn gegevens bekend over toxische effecten.

Uit een literatuur studie bleek dat twaalf secundaire metabolieten, van de 137 die door species van het genus *Fusarium* geproduceerd kunnen worden, toxisch waren in proefdieren *(Hoofdstuk 2)*. De *Fusarium* species waren uit grondstoffen voor levensmiddelen geïsoleerd. Zes van deze twaalf mycotoxinen zijn in verband gebracht met humane vergiftigingen: T-2 toxine (voedselvergiftiging en dodelijke slachtoffers), nivalenol (voedselvergiftiging), deoxynivalenol (voedselvergiftiging en immunotoxisch), acetyldeoxynivalenol (voedselvergiftiging), fumonisine B_1 (carcinogeen en in verband gebracht met slokdarmkanker in de mens) en zearalenon (oestrogeen). De overige zes mycotoxinen van de groep van 12 worden tevens beschouwd als potentieel gevaarlijk voor de mens.

De blootstelling van de consument, *exposure assessment,* werd bepaald door de analyse van granen op *Fusarium* infectie en besmetting met mycotoxinen en door de consumptie van relevante levensmiddelen te bepalen. Als gevolg van klimatologische omstandigheden varieerde de besmetting van Nederlandse granen

met *Fusarium* aanzienlijk tussen twee jaren 1991 (34 % van de monsters besmet) en 1993 (83 % van de monsters besmet) *(Hoofdstuk 3)*. Dit kan van invloed zijn op de voorkomende mycotoxinen omdat de productie van bepaalde mycotoxinen species afhankelijk zijn. Een aanzienlijke verscheidenheid in fenotype en genotype werd gevonden tussen isolaten binnen twee van de meest voorkomende *Fusarium* species *(Hoofdstuk 4)*. Uit de literatuur bleek dat de acht uit graan geïsoleerde *Fusarium* species potentieel ten minste 16 secundaire metabolieten kunnen produceren, waarvan er 6 geïdentificeerd waren als mycotoxine in de *hazard identification*. Er werd geconcludeerd dat alle potentieel geproduceerde secundaire metabolieten aanwezig kunnen zijn in granen die in Nederland geteeld worden.

Monsters van partijen graan (n=69) geoogst in Nederland in 1993 en bestemd voor de productie van levensmiddelen of diervoeders, waren bij de oogst besmet met deoxynivalenol (3 % van de monsters) of zearalenon (1 % van de monsters) boven de detectie limiet (500 ng deoxynivalenol g^{-1} en 200 ng zearalenon g^{-1} graan) *(Hoofdstuk 3)*.

Vanwege het klimaat kan maïs bestemd voor humane consumptie niet verbouwd worden in Nederland. Geïmporteerde maïs werd onderzocht op fumonisine B_1 besmetting. Achtennegentig procent (n=62) van de monsters maïs van partijen bestemd voor levensmiddelen bleek besmet te zijn met fumonisine B_1 *(Hoofdstuk 5)*. De incidentie en het besmettingsniveau met fumonisine B_1 bleken laag te zijn in levensmiddelen die maïs bevatten, ondanks de veronderstelde stabiliteit van het mycotoxine *(Hoofdstuk 6)*.

De kans werd berekend die de Nederlander loopt op de dagelijkse inname van een bepaalde hoeveelheid van fumonisine B_1 *(Hoofdstuk 7)*. Hierbij werd gebruik gemaakt van de gegevens over het voorkomen van fumonisine B_1 in maïs en de maïs opname door de bevolking. De gemiddelde maïsopname door de Nederlandse bevolking was 3 g per persoon per dag. Mensen behorende tot de groep `eaters-only` (geëxtrapoleerd uit een Canadese studie waarin deze groep werd gedefinieerd als degenen die maïsconsumptie rapporteerden tijdens het onderzoek) consumeerden gemiddeld 42 g maïs per persoon per dag. De maïsopname van mensen met een glutenintolerantie (mensen met coeliakie of de ziekte van Dühring),

de risico groep voor hoge fumonisine B_1 opname, werd geschat op 162 g per persoon per dag.

In de *hazard characterisation* werd een dosis-response toxiciteitstudie uitgevoerd met fumonisine B_1 *(Hoofdstuk 8)*. In ratten werd het toxicologische profiel vastgesteld en het doel-orgaan bij toediening van lage doses fumonisine B_1. Daarnaast werd de nadruk gelegd op de mogelijke immunotoxische effecten. De dieren werden dagelijks gedoseerd per maagsonde met, respectievelijk, 0, 0,19, 0,75 en 3 mg fumonisine B_1 kg^{-1} lichaamsgewicht. Een dosis-response gerelateerd effect werd waargenomen voor apoptose in de nieren van de ratten. Het kan niet uitgesloten worden dat chronische blootstelling aan fumonisine B_1 kan leiden tot ernstige nierschade en mogelijk een verhoogde kans op tumor ontwikkeling in dit orgaan. Het `lowest observed effect level` was 0,19 mg fumonisine B_1 kg^{-1} lichaamsgewicht. Bij hogere concentraties fumonisine B_1 werd toxiciteit waargenomen in de lever en het immuunsysteem. Een `tolerable daily intake` (TDI) van 500 ng fumonisine B_1 kg^{-1} humaan lichaamsgewicht werd berekend aan de hand van de resultaten van de toxiciteit studie.

De kans om dagelijks blootgesteld te worden aan fumonisine B_1 in de concentratie die correspondeert met de TDI werd in de *risk characterisation* geschat op 12 % voor de Nederlandse bevolking, 55 % voor de mensen behorende tot de groep van `eaters-only` en 78 % voor de mensen met glutenintolerantie (*Hoofdstuk 9)*.

De resultaten laten zien dat de gezondheid van mensen in Nederland, met name van de mensen die behoren tot de risico groep, in de huidige situatie negatief beïnvloed kan worden door mycotoxinen die door *Fusarium* geproduceerd worden en die kunnen voorkomen in levensmiddelen. Voorts kan het immunotoxische deoxynivalenol voorkomen in levensmiddelen en diervoeders waardoor blootstelling van de populatie in Nederland aan infectieziekten, met name de zoönosen, kan toenemen.

NAWOORD

Mycotoxinen zijn natuurlijke toxinen. Die positieve klank is bedrieglijk. De opname van deze natuurlijke toxinen, via voedsel en voer, kunnen zeer nadelige gevolgen hebben voor de gezondheid van mens en dier. Mycotoxinen worden niet door mensen in landbouwproducten `gestopt`. Besmetting met *Fusarium* mycotoxinen hangt af van het weer, import van granen speelt een rol, kortom de beheersbaarheid lijkt ongrijpbaar. Uitkomsten van onderzoek naar de humane blootstelling aan mycotoxinen kunnen dus gevolgen hebben voor landbouw en handel. Desondanks hebben de heren ir W. de Koe en Drs D. Kloet, van de respectievelijke ministeries van Volksgezondheid, Welzijn en Sport en Landbouw, Natuurbeheer en Visserij, opdracht gegeven tot het onderzoek dat in dit proefschrift beschreven is. Ik wil hen bedanken voor hun betrokkenheid bij het project, ik heb de uitwisseling van informatie en de openheid waarin dit gebeurde erg op prijs gesteld.

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Met zeer veel voldoening heb ik vier jaar gewerkt aan *Fusarium*.

CURRICULUM VITAE

Monique de Nijs (Wilhelmina Cornelia Maria) werd op 27 april 1965 geboren in Bergen op Zoom. Ze behaalde haar HAVO diploma in 1983 en begon in hetzelfde jaar aan haar studie tropische landbouw aan de toenmalige Rijks Hogere Landbouwschool in Deventer. Ze liep van januari tot en met augustus 1986 stage in Ethiopië bij het International Livestock Center for Africa (ILCA). In januari 1988 behaalde ze haar diploma met afstudeerrichting tropisch dierlijke productie.

In 1988 begon ze haar studie levensmiddelentechnologie aan de Landbouwuniversiteit in Wageningen (LUW). Het afstudeervak levensmiddelenchemie werd uitgevoerd aan de LUW (prof. dr ir A. G. J. Voragen en dr ir H. Gruppen). De stage werd doorlopen bij de University of California in Davis in de Verenigde Staten bij de vakgroep levensmiddelentechnologie (Prof. dr J. R. Whitaker). Aansluitend werd een afstudeervak gedaan aan de University of Guelph in Canada bij de vakgroep levensmiddelenmicrobiologie (prof. dr M. W. Griffiths en prof. dr ir F. M. Rombouts). In september 1992 behaalde ze haar diploma met afstudeerrichting levenmiddelenleer.

Van april 1993 tot en met april 1997 was ze in dienst bij de LUW, als assistent in opleiding bij de vakgroep levensmiddelentechnologie, sectie levensmiddelenchemie en -microbiologie, in opdracht van de ministeries van Volksgezondheid, Welzijn en Sport en Landbouw, Natuurbeheer en Visserij. Het in dit proefschrift beschreven wetenschappelijke onderzoek werd uitgevoerd onder leiding van prof. dr ir F. M. Rombouts en dr ir S. H. W. Notermans bij het Microbiologisch Laboratorium voor Gezondheidsbescherming, Laboratorium voor Analytisch Residu-Onderzoek en Laboratorium voor Pathologie en Immunobiologie van het Rijks Instituut voor Volksgezondheid en Milieu in Bilthoven.

Sinds 1 juli 1997 werkt ze bij TNO voeding in Zeist, divisie industriële microbiologie, afdeling voedselkwaliteit en -veiligheid.

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