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Investigations into selected mycotoxins in barley, malt and wheat

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CONTENTS

LIST OF TABLES

LIST OF FIGURES **Page**

SECTION 1. ABSTRACT

The aims of this project were to survey the incidence in UK cereal grain of certain mycotoxins which have been identified as potential concerns for European cereals and cereal products. These mycotoxins include diacetoxyscirpenol (DAS), neosolaniol, fusarenon-X, T-2 and HT-2, all of which are produced by *Fusarium* species, and citrinin, which is produced by certain *Penicillium* moulds, often in conjunction with ochratoxin A. In addition, the project investigated the potential for formation and / or degradation of these mycotoxins during malting and brewing.

Representative sample sets of barley (malting and feed), milling wheat and feed oats from the 2004 and 2005 harvest were analysed. Results suggest that the Fusarium mycotoxins neosolaniol, fusarenon-X and diacetoxyscirpenol were currently very rare in UK grown grain. The incidence of T-2 and HT-2 toxins appeared to be increasing in all cereals, but concentrations remained low, except in oats. Citrinin was not detected very frequently. However, there would appear to be a significant risk of its formation in grain which is not stored correctly.

The potential for mould growth and toxin formation during malting was investigated using artificially infected barley which had been inoculated with toxigenic moulds and held under high moisture and temperature conditions in order to encourage mould growth. The infected barley was then malted immediately. These conditions are obviously not meant to simulate commercial grain storage or commercial malting practice but were required to provide sufficient levels of infection to allow the toxins to be monitored during processing.

Results suggest that if viable mould capable of producing nivalenol or deoxynivalenol were present on barley used for malting, it is possible that mycotoxin concentrations could rise during malting. Only limited loss of these mycotoxins was observed during malting, but losses might be expected to be higher under commercial conditions, where concentrations of viable mould would be substantially lower.

In contrast, significant losses of fusarenon-X, neosolaniol, diacetoxyscirpenol, T-2 toxin and citrinin were observed during malting, even in the presence of large amounts of viable toxigenic mould. Under commercial conditions, with less mould inocula, it is probable that significant quantities of HT-2 would also be lost during malting.

Malts containing high levels of mycotoxins were brewed on the pilot scale. Brewing performance and beer quality were noticeably affected. Substantial proportions of deoxynivalenol, nivalenol, fusarenon-X and neosolaniol persisted into the final beer. However, significant losses of T-2, HT-2 and diacetoxyscirpenol were observed.

SECTION 2. SUMMARY

The aims of this project were:

1. To survey the incidence in UK cereal grain of certain mycotoxins which have been identified as potential concerns for European cereals and cereal products. These mycotoxins included diacetoxyscirpenol (DAS), neosolaniol, fusarenon-X, T-2 and HT-2, all of which are produced by *Fusarium* species, and citrinin, which is produced by certain *Penicillium* moulds, often in conjunction with ochratoxin A.
2. To compile information on moisture and temperature conditions conducive to mould growth and mycotoxin formation.
3. To assess potential for formation / degradation of the mycotoxins during malting.
4. To measure the stability of the mycotoxins towards enzyme-catalysed hydrolysis, boiling and fermentation, under conditions typically presented during the brewing process.
5. To assess the potential for carry-over of the mycotoxins into beer.

Representative sample sets of barley (malting and feed), milling wheat and feed oats from the 2004 and 2005 harvest were analysed for a range of trichothecenes, using a GC mass spectrometric method with a limit of quantification of 5µg/kg for each mycotoxin. T-2 and HT-2 toxins were also analysed by a newly developed LC mass spectrophotometric method with a lower limit of quantification (1µg/kg for HT-2 and 0.5µg/kg for T-2). Results suggest that the Fusarium mycotoxins neosolaniol, fusarenon-X and diacetoxyscirpenol, which were not detected in any of the samples tested, were currently very rare in UK grown grain. The incidence of T-2 and HT-2 toxins appeared to be increasing in all cereals, but concentrations were low, except in oats. Citrinin was not detected very frequently. However, there would appear to be a significant risk of its formation in grain which is not stored correctly, or which is stored for extended periods of time.

The potential for mould growth and toxin formation during malting was investigated using artificially infected barley which had been inoculated with toxigenic moulds and held under high moisture and temperature conditions in order to encourage mould growth. The infected barley was then malted immediately. These conditions are obviously not meant to simulate commercial grain storage or commercial malting practice, but were designed to produce barleys containing high concentrations of toxins so that partitioning during subsequent processing could be followed reliably. The relative increase or decrease of mycotoxins during the malting phase was influenced both by the capacity of the particular mould species used to flourish and produce toxins under malting conditions, and by the chemical characteristics of each mycotoxin (for example, solubility and thermal stability). Only limited loss of deoxynivalenol or nivalenol was observed during malting, but losses might be expected to be higher under commercial conditions with lower concentrations of viable mould. Results suggest that if viable mould capable of producing these mycotoxins were present on barley used for malting, then there

would be some potential for concentrations to rise during malting. Deoxynivalenol metabolites such as 3-Acetyl DON may also form during malting.

In contrast, significant loss of fusarenon-X, neosolaniol, and T-2 toxin were observed during malting, even in the presence of large amounts of viable toxigenic mould. Under commercial conditions, with less mould inocula, it is probable that significant quantities of HT-2 would also be lost during malting. Diacetoxyscirpenol and citrinin are very largely destroyed during malting.

Malts containing high levels of mycotoxins were brewed on the pilot scale. Brewing performance and beer quality were noticeably affected. Both worts and beers were distinctly cloudy, and the beers displayed a strong tendency to gush.

Substantial proportions of deoxynivalenol, nivalenol, fusarenon-X and neosolaniol persisted into the final beer, but some variations were observed, both between different toxins, and from brew to brew. All the nivalenol and fusarenone-X, and on average around 80% of the deoxynivalenol present in the malt, could be recovered in the beer. However, about 30% of neosolaniol was lost during brewing, with about one third of this being found in the spent grains. Significant losses of T-2, HT-2 and diacetoxyscirpenol were observed, and less than half of that present on the malt persisted into the beer.

SECTION 3. TECHNICAL DETAIL

1. BACKGROUND AND SCOPE OF PROJECT

The European Commission has embarked upon an extensive programme to control mycotoxins in the food chain. Legislation has already been introduced to control aflatoxins *(Reg. 257/2002)* and ochratoxin A *(Reg. 472/2002)* in cereals, and further regulations setting limits for deoxynivalenol (DON) and zearalenone (ZEA) came into force from July 2006 *(Reg 856/2005)*.The Commission is currently compiling information on the incidence of other *Fusarium* toxins (T-2, HT-2 and fumonisins) and will set limits in cereals and maize respectively by Autumn 2007. Recently the Commission has signalled its interest in four other mycotoxins; diacetoxyscirpenol (DAS), neosolaniol (NEO), fusarenon X (FUS-X) (also produced by *Fusarium* species) and citrinin (produced by *Penicillium verrucosum*, the same species which produces ochratoxin A).

The trichothecene group of mycotoxins (which includes DON, T-2, HT-2, DAS, NEO and FUS-X) are of particular concern to the cereals industry, since most of the members of this chemical group are heat stable and are therefore considered likely to persist into processed products. Some of the less common species, particularly T-2 and HT-2 toxins, are also significantly more toxic than DON, which is generally the commonest and most studied of the group. There is limited information available as to the occurrence of trichothecenes other than DON in UK grain, although very recent survey work funded by the HGCA suggests that the incidence of T-2 and HT-2 may be increasing in the UK *(HGCA Project Report No 380)*. There is also limited information on the incidence of citrinin, although the few reports which are available suggest that it frequently co-exists with ochratoxin A. There are also few published studies of mycotoxin behaviour under real processing conditions.

Scope of this project.

This project aims:

(1) to survey the incidence of the above-mentioned mycotoxins in UK grain and

(2) to explore their behaviour during processing.

The specific processes investigated were the malting and brewing processes, but since these involve a wide range of generic treatments, including washing with water, heating, enzymatic hydrolysis, solid/liquid separations and fermentation, it is likely than much of the information will be applicable to other food preparation processes.

In order to assess accurately potential partitioning into process fractions, grain with relatively high mycotoxin content is required. Sourcing naturally contaminated malting barley with the required degree of contamination would have been unreliable within the time constraints of this project, since the degree of contamination fluctuates from year to year, depending upon weather conditions. The project therefore used malting barley

which was artificially inoculated with suitable toxigenic moulds and held under conditions likely to encourage further mould growth. This grain was then used for malting and brewing trials. Thus, the results indicate how a particular mycotoxin would be likely to behave during processing if it were to be present in the starting material. They do not reflect the actual situation with regard to mycotoxin content of commercial malts and beers. Indeed, all the indications from surveillance suggest that the incidence of most of these mycotoxins in UK cereals is very low, certainly at the present time.

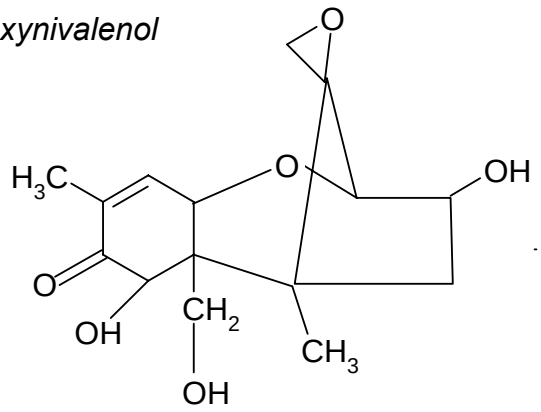
2. REVIEW OF THE LITERATURE

NEO, DAS, FUS-X and T-2 and HT-2 toxins are all mycotoxins of the trichothecene group and are produced by *Fusarium* species.

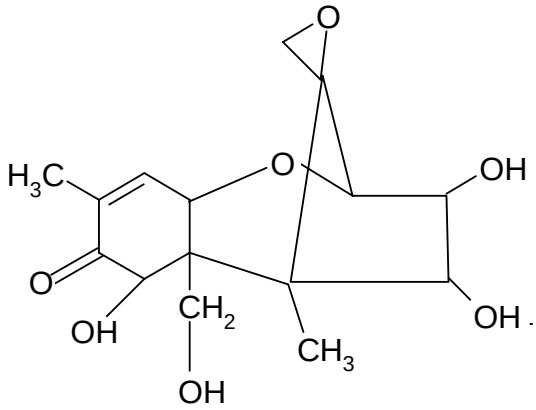
Figure 1. Structures of the trichothecene mycotoxins

Type B Trichothecenes

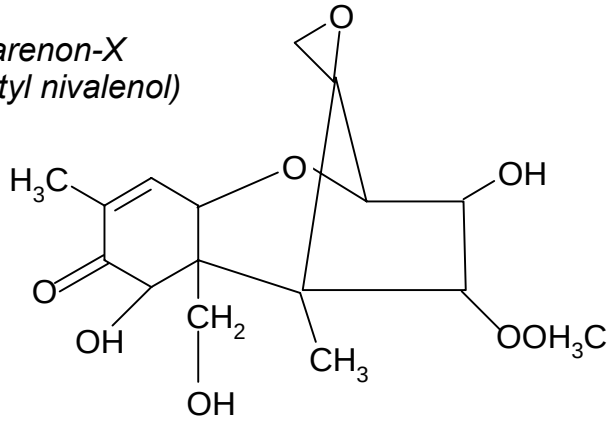
Deoxynivalenol



Nivalenol

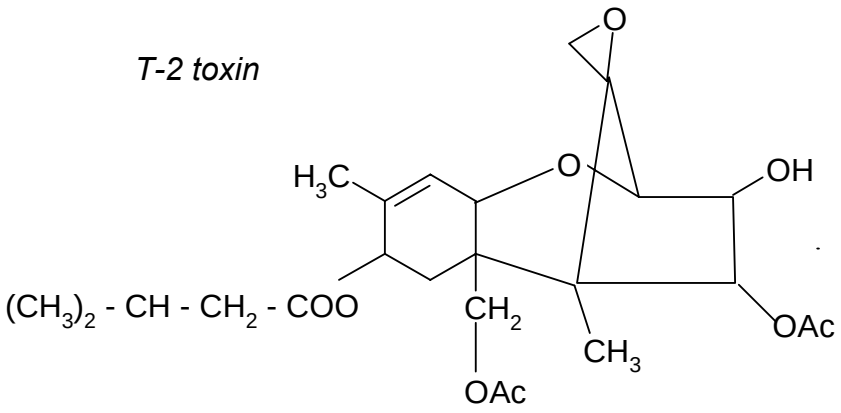


Fusarenon-X
(acetyl nivalenol)

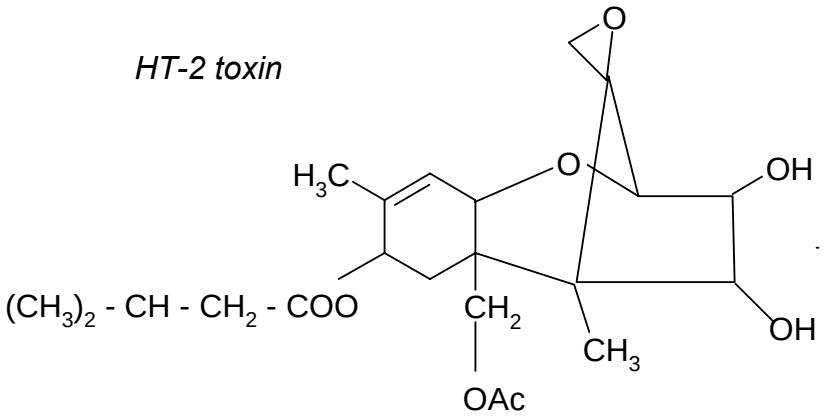


Type A Trichothecenes

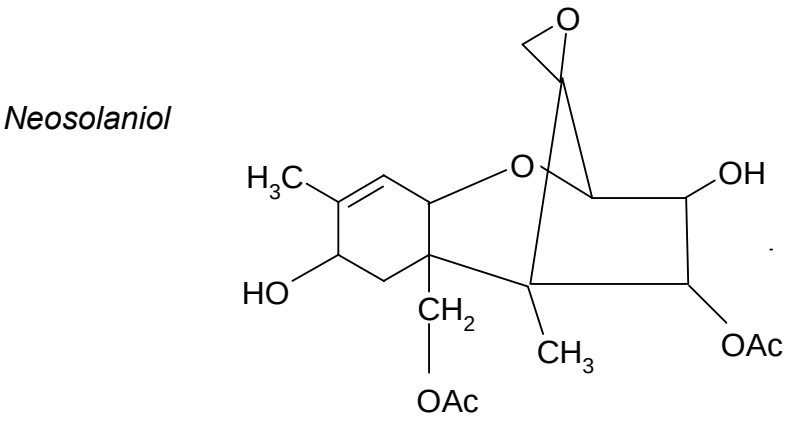
T-2 toxin



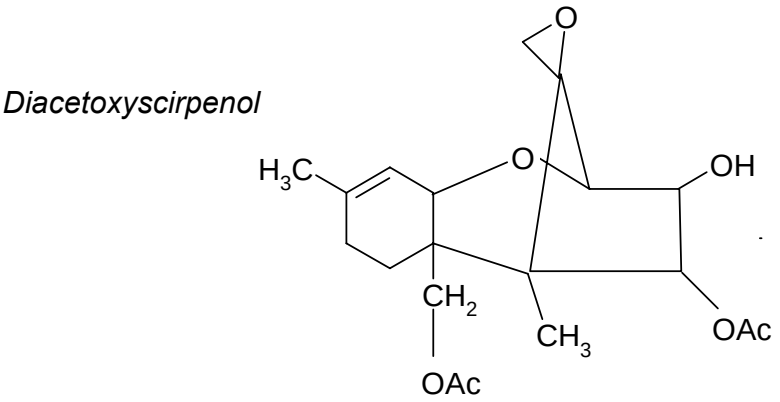
HT-2 toxin



Neosolaniol



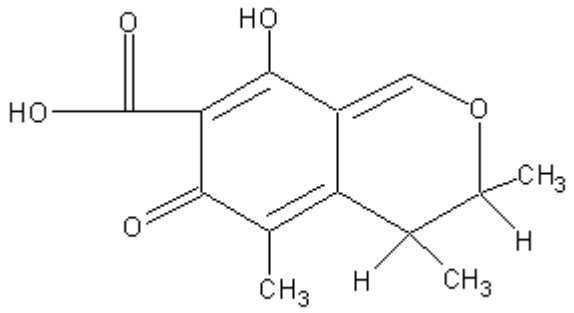
Diacetoxyscirpenol



The most commonly occurring trichothecene in Europe is DON and there is a wide literature on DON and nivalenol (NIV), which will not be reproduced here. Much less has been published concerning T-2 toxin, HT-2 toxin, FUS-X and DAS. All the trichothecene mycotoxins are based on a similar structure (see Figure 1) with an epoxide ring. Fusarenon-X (also known as 4-acetyl nivalenol) resembles DON and NIV in that it is a type B trichothecene, with a carbonyl group in the C-8 position. NEO, DAS, T-2 toxin and HT-2 toxin lack this carbonyl group and are classified as Type A trichothecenes.

Citrinin is not a *Fusarium* toxin and is quite different structurally to the trichothecenes. The structure is shown in Figure 2.

Figure 2. Structure of citrinin



Mould species

Neosolaniol (NEO) is thought to be produced mainly by *F. poae* and *F. sporotrichiodes (Osborne, 1988; WHO, 1990)*, both of which can occur on UK grain *(Osborne, 1988)*. *F. sporotrichiodes* is also a major producer of T-2 and HT-2 toxins. Diacetoxyscirpenol (DAS), which is one of a large group of related scirpenols, which also includes scirpentriol (STO), triacetoxyscirpenol (TAS), and several mono- and di-acetoxy compounds (3,4-DAS; 3,15-DAS; 15-MAS; 4-MAS; 3-MAS). DAS is also thought to be produced by *F. poae* and *F. sporotrichiodes* (*WHO, 1990),* as well as by *F scirpi.* Fusarenon X (FUS-X) is thought to be produced by *F. graminearum* (*WHO, 1990)*.

Citrinin is produced by *Penicillium* (*P. expansum, P. citrinin* and *P. verrucosum*) and *Aspergillus* species, and often co-occurs with ochratoxin A (OA) in cereals and with patulin in fruit. The *Penicillium* species can flourish under relatively low moisture conditions, consequently citrinin and OA are commonly formed in grain storage, where other moulds which would normally compete with them, such as the Fusarium species, are less able to grow.

Toxicity

The trichothecenes all exert similar toxic effects on animals *(European Commission, 2002)*, with the main effects being:

- Inhibition of protein synthesis by binding to ribosomes
- Inhibition of RNA and DNA synthesis
- Toxic effects on cell membranes
- Induction of apoptosis

Trichothecenes are therefore particularly toxic for actively dividing cells, such as those in the lymphatic and haematopoietic tissue, and in epithelial membranes, for example in the intestines. The different toxins vary significantly in their toxic potencies (Table 1) and it is still not clear whether their mechanisms of action are identical at a biochemical level.

Table 1. Toxicity of trichothecenes and citrinin

Toxin	**Oral LD 50 (µg/kg body weight)**	**TDI (µg/kg body weight)**
DON	40 – 78[2]	1[2]
NIV	19 – 40[2]	0.7[2]
DAS	23[1]	
NEO	15[1]	
HT-2	9[1]	
T-2	5.2[1]	Combined t-TDI = 0.06[2]
Citrinin	Oral 50[3] Subcutaneous 67[4]	
Ochratoxin A		Tolerable Weekly Intake 120 ng/kg body weight [5]

(Sources: [1] European Mycotoxin Awareness Network: [2] European Commission, 2002:
[3] EMAN network: [4] Fungal research Trust, 2006: [5] EFSA, 2006:

The European Commission's Scientific Committee on Food *(European Commission, 2002)* found insufficient evidence of a common mechanism for them to set a group Tolerable Daily Intake (TDI) for all the trichothecenes, although a combined TDI has been set for the total of T-2 and HT-2. As is evident from Table 1, T-2 and HT-2 are the most toxic of the commoner trichothecenes, but DAS, NIV and NEO are all significantly more toxic than DON.

Citrinin is a nephrotoxin which can act synergistically with OA *(Abramson, 1991)* and it also has embryotoxic effects *(Vesela, 1983)*. Citrinin is reported to be significantly less cytotoxic than OA *(Kitabatake, 1993)*.

However, in *in vitro* studies with human cell lines, citrinin was found to have similar genotoxic potency in OA, albeit via a different mechanism *(Knasmuller, 2004)*.

Occurrence

In 2003 the European Commission published a SCOOP report on the occurrence of trichothecenes in foodstuffs in the EU *(European Commission 2003)*. The samples tested included raw cereal grains (barley, wheat, rye, oats) as well as processed products such as wheat flour, malt, and consumer products such as bread, beer and cakes. A summary of the results is shown in Table 2, and these suggest that while DON was detected in 57% of samples, and T-2 and HT-2 in 14-20% of samples, FUS-X was found in only 10% and NEO and DAS in less than 5% of samples. Table 3 shows more detailed data, again from the SCOOP report, for the countries reporting positive samples, and the cereal matrixes in which the individual toxins were found. There are, however, some limitations to this collation of data. Although most Member States submitted data for DON and several submitted data for nivalenol, T-2 and HT-2, only 2 or 3 countries analysed samples for FUS-X, DAS and NEO. Most information is available for France. There were also wide variations in the limits of detection achieved by the different countries, ranging from 5 (the limit of detection for the current study) to as high as 90µg/kg. Also, it should be remembered that this data will relate to cereals harvested prior to 2001 at the latest. Data presented in the current study for T-2 and HT-2 in UK cereals suggests that the incidence of *Fusarium* toxins is currently undergoing a period of change in Western Europe.

Table 2. Summary of Fusarium toxin occurrence data from EU Member States
(Source: SCOOP report of Fusarium toxins, European Commission 2003).

***Fusarium* toxin**	**No. of Countries reporting**	**No. samples**	**% of positive samples**
Type B trichothecenes			
DON	11	11,022	57
NIV	7	4,166	16
3-Ac DON	6	3,721	8
15-Ac DON	3	1,954	20
Fusarenon-X	3	1,872	10
Type A trichothecenes			
T-2 toxin	8	3,490	20
HT-2 toxin	6	3,032	14
Neosolaniol	2	1,323	1
DAS	3	1,886	4

Table 3. Occurrence of individual mycotoxins in cereal matrices in EU Countries

Mycotoxin	Reporting country	% of positive samples	Matrices	Maximum µg/kg
FUS-X	France	18	Wheat, maize	50
	Denmark	20	Wheat flour, rye flour	193
T-2	Finland	<1	Malt, oats, rye	547
	France	19	Wheat, wheat flour, maize	50
	Italy	96	Wheat, wheat flour, barley, maize, semolina	280
	Norway	10	oats	317
	Austria	20	Maize, oats	1150
HT-2	Denmark	20	Wheat flour, rye flour	70
	Finland	5	Wheat, barley, malt, oats	822
	France	19	Maize, oats	50
	Norway	16	Wheat, oats	711
	UK	<1	Wheat, maize, barley	77
DAS	France	10	Wheat, maize, barley	50
NEO	France	1	Wheat, maize	40

This data suggests that DAS, NEO and FUS-X are currently very rare in the EU. However, DAS and FUS-X occurred in about 10% of samples where this toxin was tested for. Given the infrequent testing, it is probable that incidence in the EU could be under-reported.

P. citrinin is very widely distributed and has been isolated from most foodstuffs, with the major sources being whole cereals, particularly rice, maize and wheat, and cereal flours (*Pitt, 1989)*. Infection of rice is characterized by the development of a characteristic yellow colour, and indeed it was an investigation into the causes of "yellow rice disease" which led to the earliest appreciation of the potential toxicity of common moulds *(Zasshi, 2004)*. A review commissioned by the HGCA some years ago *(Osborne, 1988)* concluded that citrinin was one of the most frequently detected mycotoxins in UK grain. Scudamore reported that, out of 18 samples of UK grain which contained OA, 16 also tested positive for citrinin, and in 4 of these samples the level of citrinin greatly exceeded that of OA *(Scudamore, 1993)*. In a recent survey of breakfast cereals in France, 20% of samples contained detectable citrinin (69% contained OA), at concentrations of 1.5 – 42µg/kg *(Molinid, 2005)*. Samples of grain dust from wheat storage facilities in Belgium were also found to contain both citrinin and OA, at concentrations of 137 - 344 and 17 - 318µg/kg respectively *(Tangni, 2006)*. Testing for citrinin has, however, more frequently been focussed on areas where a relevant mycotoxicosis has been reported. Citrinin, together with OA, was found in 3 – 14% of cereal samples collected from villages in Bulgaria associated with outbreaks

of Balkan endemic nephropathy, at mean concentrations of between 6 and 180µg/kg *(Vrabcheva, 2000)*. Citrinin levels in these samples were found to be significantly higher than concentrations of OA.

Behaviour during processing

Little information is available concerning the behaviour of these mycotoxins during processing. Most trichothecenes are expected to survive processing since they are relatively heat stable and water soluble. Inter-conversion via acetylation / deacetylation reactions forming acetylated analogues is well recognised for DON, and similar reactions have also been reported for DAS *(Perkowski, 2003*). This may be of particular relevance to enzymatically active processes such as malting and brewing.

Citrinin is reported to be unstable during processing *(Leatherhead RI, Citrinin fact sheet)* but no original references are given in this publication. One report of malting and brewing with barley heavily contaminated with citrinin and OA *(Krogh, 1974)* suggests that both are largely destroyed during processing. However, the limits of detection of the analytical methods used in this old study were much higher than those available today, and more recent brewing studies have confirmed that at least one third of OA in malt can survive into the beer *(Baxter, 2001)*. It is therefore possible that significant quantities of citrinin might also survive the malting and brewing processes. However, there have been no more recent published studies of citrinin during brewing.

Many published studies suggest that citrinin can be detoxified by heating. Citrinin decomposes when heated at 140°C in semi-moist condition, but both toxic *(Trivedi, 1993)* and non-toxic *(Hirota, 2002)* breakdown products have been reported. Other workers have observed detoxification at lower temperatures *(Kirby, 1987),* so the potential toxicity of citrinin in processed foods remains unclear.

3. MATERIALS AND METHODS

3.1 Samples collected for surveillance

Sample sets collected as part of the HGCA projects 2804 (Review of food safety issues relating to the supply and market acceptability of UK malting barley and UK malt), 2819 (milling wheat) and 3033 (Monitoring the quality and safety of grain and grain-derived co-products destined for animal feed) were utilised to survey the occurrence of the mycotoxins of interest in commercial samples of UK grain.

Malting barley (Project 2804) *(HGCA Project Report No. 380)*

Representative sets of samples were collected for Project 2804 between 2003 and 2005 with the collaboration of the Maltsters Association of Great Britain (MAGB), whose members represent the majority of commercial maltsters in the UK. The sample sets from 2004 and 2005 were also used for this current project.

- Samples of freshly harvested malting barley from the 2005 harvest were collected in September-October of the same year. This sample set consisted of 18 freshly harvested barleys, drawn from all members of the MAGB, with the number of samples per company governed by its production volumes. The companies were instructed to take several sub-samples from the bulk and to mix them to produce the final sample submitted.
- Samples of stored malting barleys from the 2004 and 2005 harvests, also drawn from all members of the MAGB, were collected in April-May of the following year. Ten kilogramme samples were taken according to the protocol set down in Directive 2002/26 for the official control of ochratoxin A in foods. Each set comprised about 20 samples of stored for at least 6 months.

Details of the sample sets are given in Appendix 1.

Feed cereals (barley, oats) (Project 3033) *(HGCA Report No. 387)*

Two sample sets from this project were analysed for *Fusarium* toxins:

- Freshly harvested samples of feed barley and oats/oatfeed from the 2004 harvest, collected September-December 2004.
- Freshly harvested samples of feed barley and oats from the 2005 harvest, collected September-November 2005.

Details of the sample sets are given in Appendix 2.

Milling wheat (Project 2819)

A set of freshly harvested milling wheats from the 2004 harvest was collected by nabim (National Association of British and Irish Millers) as part of Project 2819, and a portion of each sample was sent to BRi. Details of the sample set are given in Appendix 3.

3.2 Analysis of mycotoxins

- **Trichothecenes**

FUS-X, NEO and DAS were analysed by extending the BRi in-house procedure for trichothecene analysis, which is based on a published method *(Patel et al, 1996).* The mycotoxins were extracted using acetonitrile/water, partially purified using trichothecene clean-up columns, then derivatised and analysed by GC-mass spectrometry. The limit of quantification for each toxin was 5µg/kg. BRi participates in FAPAS proficiency tests for trichothecenes. Z scores are available on request.

In 2006, an LC.MS.MS method was developed for T-2 and HT-2. Samples were extracted with methanol/water and a portion of the extract loaded onto commercially available immuno-affinity columns (BioPharm). The columns were eluted with methanol and the eluate analysed by LC.MS.MS using selected ion monitoring (ion 327 for T2 and 285 for HT-2). This method achieved very low limits of detection (1µg/kg for HT-2 and 0.5µg/kg for T-2.

- **Citrinin**

Samples were milled and extracted with a dichloromethane/phosphoric acid mixture. The extract was partially purified using a polyamide SPE column and eluted with methanol/formic acid. The eluate was concentrated and analysed by LC-MS-MS. The limit of quantification was 0.1µg/kg.

- All mycotoxin results are reported corrected for recovery.

3.3 Production of contaminated barleys

In order to follow the behaviour of the mycotoxins during processing, raw grain containing substantial quantities of toxin was required. The surveillance indicated that the levels of these mycotoxins in UK grain are currently very low (at least in the harvests investigated). Consequently it was necessary to produce artificially contaminated barley bulks.

3.3.1 Mould cultures

Cultures of *Fusarium* species thought to produce the mycotoxins of interest were obtained from VTT and from CABI Biosciences. *Penicillium verrucosum* and *Penicillium citrinin* were obtained from CABI Biosciences. Cultures were inoculated into 10ml YM broth and incubated in stirred flasks at 25°C for 24 hours. This liquid was then added to a fresh YM broth (100ml) and incubated under the same conditions for a further 24 hours. The resulting solution was transferred to a larger volume of YM broth (1 or 10 litres as required) and incubated for a further 2-4 days.

3.3.2 Inoculation of barley

In order to determine the best conditions for mycotoxin production small batches of barley were inoculated with the relevant mould then held under different conditions before testing for mycotoxin content. The mould culture (20, 40 or 60ml, giving a moisture content in the barley of approximately 19, 25 or 31% respectively) was added to samples of BRi's stock malting barley (250g; variety Pearl) in wide-necked glass jars fitted with muslin covers over the necks. The jars were shaken well to disperse the liquid, then held for approximately 4 weeks at the specified temperature (18°C; 20°C; 28°C). Samples were removed at intervals and frozen before analysing for the relevant mycotoxins. In some cases mould growth was also assessed visually.

The incubation conditions which resulted in the highest concentrations of the mycotoxin of interest were then simulated on a larger scale to produce sufficient barley for malting. Barley (50kg) was contained one of BRi's drum malting vessels and the mould culture, in 10 litres of liquid, sprayed onto the grain using the integral spray nozzle fitted in the drum. The drum was then rotated several times in order to mix the culture and the grain. The drum was then held at the specified temperature for one month to allow mould growth and toxin formation.

Appropriate health and safety precautions were taken, with a personal protection suit being worn during application of the culture and during sampling.

3.4. Pilot scale malting

A sample of the contaminated barley was taken prior to malting and stored frozen. The contaminated batch was then malted using a standard protocol for that variety, except that steeping conditions were modified to take into account the high starting moisture (Table 4).

Table 4. Inoculation and malting conditions for pilot scale malting studies

Stage	Conditions
Inoculation	Pearl barley (50 kg) 10 litres mould culture Held at 20°C for 4 weeks
Steeping	8 hours wet ; 14 hours air rest 10 hours wet ; 10 hours air rest 4 hours wet ;2 hours air rest 16 – 20 °C
Germination	4 days at 16 - 19°C (1°C rise per day)
Kilning	10 hours with 100% fresh air, air-on temperature 40°C rising to 55°C 2 hours 65% fresh air, 35% re-circulated air, air-on 60 °C 3.5 hours 60% fresh, 40% re-circulated air, air-on 60°C 5 hours 10% fresh air, 90% re-circulated air, air-on 65°C, rising to 80°C 5 hours 90-100% re-circulated air, air-on 85°C

Samples were taken throughout processing and stored frozen prior to toxin analysis. It should be noted that, although every attempt was made to obtain a representative sample by taking sub-samples from different parts of the bulks and combining them, the total size of the sample removed was necessarily limited. Given the recognised in-homogeneity of mould growth and toxin development, some variation between samples is inevitable.

3.5 Pilot scale brewing

The contaminated malts were then brewed in BRi's pilot brewery (1 hectolitre scale). A standard lager brewing protocol (shown in Table 5) with a fixed temperature infusion mash at 65°C was used for each brew. Samples were taken throughout processing (also shown in Table 5) and stored frozen prior to being analysed for mycotoxins using the GC.MS method with a limit of detection of 5µg/kg. Mash and trub samples, which were in the form of thick slurries, were centrifuged prior to analysis and the solid and liquid fractions analysed separately.

Table 5. Pilot brewing conditions

Brewing Stage	Conditions	Samples taken
Grist:	15 kg test malt liquor/grist ratio 3:1	Milled malt grist
Mashing	Infusion mash at 64°C for 60 minutes. Sparge temperature 78°C	Sample of whole mash taken and centrifuged: insoluble and soluble portions analysed separately
Wort separation	Pilot scale lauter Approximate lautering time 80-90 minutes.	Sweet wort Spent grains
Kettle Boil	Boil time 90 minutes Hop grist; 12.5g $HOPCO_2N$ at start of boil. 20g Saaz hop pellets after 80 minutes boiling.	Wort, pre-boil (after addition of hops) Wort, post boil
Whirlpool	Duration approximately 30 minutes ; hot	Wort, post whirlpool Trub
Fermentation	12°C for 6 days or until PG < 1010° gravity Yeast strain BRYC 32	Pitching wort Fermenting wort (sampled every other day)
Racking	Flocculated yeast drawn off from beer	Beer at rack Spent yeast
Maturation	3 days at 13° 1-2 days cold rest at 3°C Yeast harvested Minimum of 7 days cold maturation at 0°C	
Packaging	DE filter sheets, type XE 200 + XE5 275ml bottles	Beer pre-pasteurisation
Pasteurisation	15 min at 60°	Bottled beer

3.6. Sampling and reproducibility - limitations

The heterogeneity of mycotoxin formation in mould-infected grain is well recognised. This is likely to have been amplified during the current investigations since the mould cells in the inoculum applied to the grain tended to form clumps. While every effort has been made to obtain representative samples by collecting several sub-samples and combining these, it is probable that these would not always be truly representative.

Samples taken during processing were of necessity limited in size, so it was not possible to collect very large samples, as recommended by EU sampling protocols for mycotoxins. Mash, spent grains and fermenting wort are also inherently heterogeneous, since they consist of mixtures of relatively coarse solid material suspended in liquid. Where necessary, these samples were centrifuged prior to analysis and the liquid and solid fractions analysed separately. Because of these limitations, some variation in toxin concentrations between individual samples taken during processing is not surprising. However, the final beer can be regarded as homogenous, thus it is possible to gain a relatively good indication of the overall extent of mycotoxin carryover into the final product.

4. RESULTS

4.1. Surveillance of UK cereals

4.1.1. Trichothecenes

Malting Barley: Table 6 shows the concentrations of trichothecenes in malting barleys and malts from the 2004 and 2005 harvests.

Table 6. *Fusarium* mycotoxins in malting barleys and malts

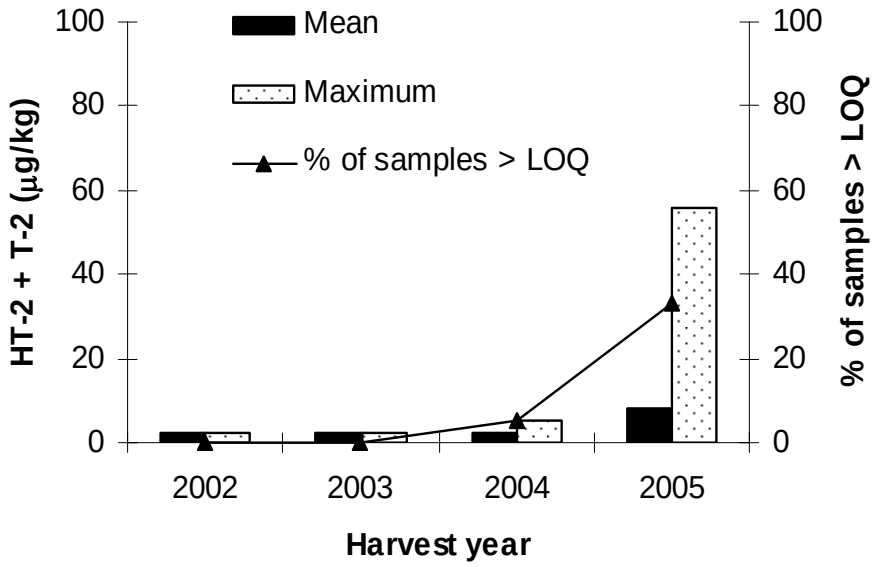
Mycotoxin *(LOQ is 5µg/kg for all toxins except where otherwise stated. Mean calculated by assuming that samples less than LOQ = half LOQ)*	**Mycotoxin concentration (µg/kg)**				
	2004 Stored barleys *N = 20*	**2004 Malts from stored barleys** *N = 20*	**2005 Freshly harvested barleys** *N = 18*	**2005 Stored barleys** *N = 18*	**2005 Malts from stored barleys** *N = 18*
DON					
Mean (µg/kg)	6.9	4.0	3.3	8.3	4.3
Maximum (µg/kg)	28	17	16	33	10
% of samples > LOQ	40	20	6	33	39
NIV					
Mean (µg/kg)	5.6	2.5	2.8	4.8	3.4
Maximum (µg/kg)	8	<5	8	12	12
% of samples > LOQ	11	0	6	13	11
HT-2					
Mean (µg/kg)	4.3	2.7	6.1	4.1	0.74
Maximum (µg/kg)	38	7	35	7.4	3.5
% of samples > LOQ	5	5	33	94	11
T2					
Mean (µg/kg)	3.7	2.7	3.6	1.3	0.54
Maximum (µg/kg)	26	7	19	2.4	5.4
% of samples > LOQ	5	5	11	89	6
HT-2 + T2					
Mean (µg/kg)	5.7	3.3	8.1	5.3	1.3
Maximum (µg/kg)	64	14	56	9.3	8.9
% of samples > LOQ	5	6	33	94	11
13- + 15-acetyl DON					
Mean (µg/kg)	2.5	2.5	2.5	2.5	2.5
Maximum (µg/kg)	<5	<5	<5	<5	<5
% of samples > LOQ	0	0	0	0	0
Neosolaniol					
Mean (µg/kg)	2.5	2.5	2.5	2.5	2.5
Maximum (µg/kg)	<5	<5	<5	<5	<5
% of samples > LOQ	0	0	0	0	0
DAS					
Mean (µg/kg)	2.5	2.5	2.5	2.5	2.5
Maximum (µg/kg)	<5	<5	<5	<5	<5
% of samples > LOQ	0	0	0	0	0
Fusarenon-X					
Mean (µg/kg)	2.5	2.5	2.5	2.5	2.5
Maximum (µg/kg)	<5	<5	<5	<5	<5
% of samples > LOQ	0	0	0	0	0

Limit of quantification: T-2 = 0.5 µg/kg; HT-2 = 1.0 µg/kg

FUS-X, NEO and DAS were not detected in any of the samples from either harvest. Likewise, the acetylated DON species could not be detected in any sample. From 2006 onwards the more sensitive LC.MS.MS method was used for T-2 and HT-2, and the values shown in Table 6 were obtained using this method. As would be expected, the incidence is very much higher than with the less sensitive GC.MS method, and the results suggest that traces of T-2 and HT-2 are widespread in most samples, with the concentrations of T-2 being slightly lower than those of HT-2. The results also suggest that there is a net loss of T-2 and HT-2 in commercial malting.

Figure 3 shows data for freshly harvested barleys in 2005 compared with those from previous harvests (reported under Project 2804). All samples were analysed using the GC.MS method with a limit of detection of 5µg/kg. T-2 toxin and HT-2 toxin are expressed as the sum of the two species, since it is probable that this is how the EU limit will be expressed. It is evident that there is a significant increase in the incidence of these two trichothecenes in 2005, although the actual concentrations remain very low.

Figure 3. Sum of T-2 + Ht-2 toxins in freshly harvested malting barleys



Feed barleys: Results for the feed barleys (Table 7) were similar to those for malting barleys. None contained detectable NEO, DAS or FUS-X, but, of the samples from the 2005 harvest, almost half contained detectable HT-2 or T-2 toxin, at concentrations similar to those in malting barleys.

Table 7. *Fusarium* mycotoxins in feed barley from the 2004 and 2005 harvests

Mycotoxin *(Limit of quantification is 5 µg/kg for all mycotoxins).*	**Mycotoxin concentration (µg/kg)** **2004** *Number of samples = 26*	**2005** *Number of samples = 21*
DON		
Mean (µg/kg)	9.4	12
Maximum (µg/kg)	55	91
% of samples > LOQ	46	29
NIV		
Mean (µg/kg)	6.5	4.8
Maximum (µg/kg)	38	18
% of samples > LOQ	31	19
HT-2		
Mean (µg/kg)	3.8	7.2
Maximum (µg/kg)	35	30
% of samples > LOQ	4	43
T2		
Mean (µg/kg)	4.1	2.8
Maximum (µg/kg)	44	8
% of samples > LOQ	4	5
HT-2 + T2		
Mean (µg/kg)	7.8	10
Maximum (µg/kg)	79	33
% of samples > LOQ	4	43
13- + 15-acetyl DON		
Mean (µg/kg)	2.5	2.5
Maximum (µg/kg)	<5	<5
% of samples > LOQ	0	0
	Number = 14	*Number = 21*
Neosolaniol		
Mean (µg/kg)	2.5	2.5
Maximum (µg/kg)	<5	<5
% of samples > LOQ	0	0
DAS		
Mean (µg/kg)	2.5	2.5
Maximum (µg/kg)	<5	<5
% of samples > LOQ	0	0
Fusarenon-X		
Mean (µg/kg)	2.5	2.5
Maximum (µg/kg)	<5	<5
% of samples > LOQ	0	0

4.1.1.2 Milling wheat

Twenty-five samples of milling wheat from the 2004 harvest were analysed for trichothecenes using the GC.MS method with a limit of quantification of 5µg/kg. The results are shown in Table 8.

Table 8. ***Fusarium*** **mycotoxins in milling wheat from the 2004 harvest**

Mycotoxin *Number of samples = 25*	**Mycotoxin concentration µg/kg**
DON	
Mean (µg/kg)	92
Maximum (µg/kg)	396
% of samples > LOQ	88
NIV	
Mean (µg/kg)	13
Maximum (µg/kg)	68
% of samples > LOQ	60
HT-2	
Mean (µg/kg)	7.3
Maximum (µg/kg)	78
% of samples > LOQ	16
T2	
Mean (µg/kg)	2.5
Maximum (µg/kg)	<5
% of samples > LOQ	0
HT-2 + T2	
Mean (µg/kg)	8.1
Maximum (µg/kg)	81
% of samples > LOQ	16
13- + 15-acetyl DON	
Mean (µg/kg)	2.8
Maximum (µg/kg)	7
% of samples > LOQ	4
Neosolaniol	
Mean (µg/kg)	2.5
Maximum (µg/kg)	<5
% of samples > LOQ	0
DAS	
Mean (µg/kg)	2.5
Maximum (µg/kg)	<5
% of samples > LOQ	0
Fusarenon-X	
Mean (µg/kg)	2.5
Maximum (µg/kg)	<5
% of samples > LOQ	0

The results show that, as with barley, NEO, FUS-X and DAS were not detected in any samples. Traces of 15-acetyl DON were found in one sample. A significant proportion of samples (16%) contained HT-2 at concentrations in excess of 5µg/kg, but T-2 toxin was not detected. As expected, DON was detected in the majority of samples.

4.1.1.3. Oats

Table 9. *Fusarium* mycotoxins in feed oats and oatfeed from the 2004 and 2005 harvests

Mycotoxin	Mycotoxin concentration (µg/kg) 2004 *Number of samples = 7*	2005 *Number of samples =8*
DON		
Mean (µg/kg)	19	11
Maximum (µg/kg)	63	25
% of samples > LOQ	57	50
NIV		
Mean (µg/kg)	107	53
Maximum (µg/kg)	359	196
% of samples > LOQ	86	63
HT-2		
Mean (µg/kg)	660	940
Maximum (µg/kg)	2453	1602
% of samples > LOQ	100	100
T2		
Mean (µg/kg)	161	341
Maximum (µg/kg)	547	453
% of samples > LOQ	100	100
HT-2 + T2		
Mean (µg/kg)	822	1281
Maximum (µg/kg)	3000	2142
% of samples > LOQ	100	100
13- + 15-acetyl DON		
Mean (µg/kg)	2.5	2.5
Maximum (µg/kg)	<5	<5
% of samples > LOQ	0	0
	Number = 5	*Number = 8*
Neosolaniol		
Mean (µg/kg)	4	36
Maximum (µg/kg)	10	91
% of samples > LOQ	20	50
DAS		
Mean (µg/kg)	2.5	2.5
Maximum (µg/kg)	<5	<5
% of samples > LOQ	0	0
Fusarenon-X		
Mean (µg/kg)	2.5	2.5
Maximum (µg/kg)	<5	<5
% of samples > LOQ	0	0

Unlike barley and wheat, all the oats and oat feed samples contained high concentrations of T-2 and HT-2 toxins, and several samples from both harvests also contained NEO, although at somewhat lower concentrations (Table 9). As with barley and wheat, HT-2 was present at significantly higher concentrations than was T-2 toxin

(generally between 2 and 4 times higher). There was a trend for concentrations of both T-2 and HT-2 to be slightly lower in whole oats than in oatfeed, but insufficient samples were tested for the difference to be statistically significant.

4.1.2 Citrinin

Citrinin is produced by the same moulds as is OA, therefore could be expected to occur relatively frequently in stored cereals. However, it is rarely tested for and analysis can be problematical.

Table 10. Citrinin in stored barley and wheat

Limit of quantification 0.1 (µg/kg)	**Citrinin concentration (µg/kg)**				
	Barley		**Malt**		**Wheat**
	2003	**2004**	**2003**	**2004**	**2004**
Number of samples	5	19	5	19	25
Mean (µg/kg)	0.18	0.2	0.44	0.2	0.29
Maximum (µg/kg)	0.7	3.0	2.0	2.9	4.5
Incidence (% > 0.1 (µg/kg)	20	5	20	5	8

Samples of malting barley, malts and milling wheat from the 2003 and 2004 harvests were analysed for citrinin (limit of quantification, 0.1µg/kg) (Table 10). Citrinin was detected in 1 out of 5 barley samples (20%) from the 2003 harvest and one out of 5 malt samples (not the same batch as the barley). Both of these samples also contained OA. Although this incidence of citrinin appears relatively high, it should be noted that these samples had been stored for more than a year after harvest and are unlikely to be typical of commercial samples. Also the number of samples tested was very small. It may be worth noting, however, that even after this extended period of storage citrinin concentrations were no higher than those found in samples from subsequent year.

In the 2004 samples, (which had been stored for approximately 9 months after harvest) incidence in both barley and malt much lower – around 5%. Incidence in wheat was similar, at 8%. Citrinin was detected in one barley and also in the malt made from that barley. Ochratoxin A was detected in 16% of the barleys and 52% of the malts in the same sample set. The barley sample which was positive for citrinin did not contain detectable OA, although the malt did contain low levels. These results suggest that there is no clear relationship between the incidence of OA and that of citrinin, although the number of samples is too small to draw any definitive conclusions.

4.2. Mould growth and mycotoxin formation in inoculated barley

Initially, attempts were made to produce the toxins of interest using *Fusarium* moulds which had been isolated from barley. In the first experiment, 250g samples of malting barley were inoculated with cultures of *F. graminearum* (VTT reference number D-95470), *F. culmorum* (VTT reference number D 80148 : isolated from barley), *F. sporotrichioides* (VTT reference number D-72014 : isolated from grain) and *F. poae* (VTT reference number D82182 : isolated from oats), kindly provided by VTT Technical Research Centre, Finland. The mould culture was applied at three different rates, and the inoculated barleys incubated at 16, 20 or 28°C. Results for mould growth and trichothecene content after 4 weeks incubations are shown in Tables 11 and 12. Although mould growth was clearly visible with several of the samples, there was little correspondence between mould growth and toxin concentration.

4.2.1 *F. graminearum, F. culmorum* and *F. poae*

The sample inoculated with *F. graminearum* did not show any visible mould growth except at 28°C, with small quantities of DON being present in most incubations.

With *F. culmorum* there was visible mould growth and some DON formation in most samples, while *F. poae* showed little growth under any of the conditions tested.

4.2.2 *F. sporotrichiodes*

The samples inoculated with *F. sporotrichiodes* also showed substantial mould growth at 28°C, but little toxin development at this temperature. In contrast, at 20°C significant concentrations of T-2 and HT-2 toxins were formed, and neosolaniol was also present. These conditions were selected for a pilot scale study.

Significant concentrations of FUS-X or DAS were not found for any of the inoculations.

Further inoculations were therefore carried out using Fusarium species purchased from CABI Biosciences. These species were reported to produce the toxins of interest, although they were not necessarily isolated from cereals. Results are shown in Table 13 and 14.

4.2.3 *F. scirpi* and .*F crookwellense*

The inoculation with *F. scirpi* (IMI 112503 : isolated from barley seedlings in Germany) showed significant development of DAS, together with small amounts of NEO at 16-20°C. This mould produced negligible amounts of other trichothecenes such as DON, NIV, T-2 or HT-2.

F. crookwellense (IMI 334774 : isolated from potato tubers) displayed significant formation of FUS-X, again at 16-20°C, associated with significant NIV but no appreciable formation of DON, T-2 or HT-2 .

These conditions were therefore also selected for pilot scale studies.

4.2.4 *P. verrucosum* and *P. citrini*

Similar incubations were carried out with cultures *P. verrucosum* (IMI 297964: isolated from Madeira cake) and *P. citrinin* (IMI 091961), also from CABI Biosciences, in order to obtain barley with a high level of citrinin. Results are shown in Table 14. Both species resulted in the formation of significant quantities of citrinin, but *P. verrucosum* gave higher levels and was chosen for pilot scale studies.

Significant formation of trichothecenes would not be expected with Penicillium inoculations, and indeed no DAS, NEO, FUS-X, NIV, T-2 or HT-2 was detected. However, DON was found in most samples, albeit at low levels. This is likely to be due to natural mould inoculum on the starting barley.

Table 11. Mould growth and mycotoxin formation (*F. graminearum* and *F. sporotrichiodes from VTT*)

(Mycotoxin concentrations are expressed as µg/kg as is)

Sample No.	Micro-organism	Inoculum rate	Incubation Temp	Visual growth	DAS	Neosolaniol	Fusarenon X	DON	3-Ac DON	15-Ac-DON	NIV	HT-2	T-2
1	Fusarium graminarium	x1	16	-	<5	<5	<5	<5	<5	<5	<5	<5	<5
2		x2	16	-	<5	<5	<5	**21**	<5	**50**	<5	<5	<5
3		x3	16	-	<5	<5	<5	<5	<5	<5	<5	<5	<5
4		x1	20	-	<5	<5	<5	**6**	<5	<5	<5	<5	<5
5		x2	20	-	<5	<5	<5	**47**	<5	**90**	<5	<5	<5
6		x3	20	-	<5	<5	<5	**4**	<5	<5	<5	**19**	<5
7		x1	28	1+	<5	<5	<5	**3**	<5	<5	<5	**8**	**10**
8		x2	28	1+	<5	<5	<5	**62**	<5	**29**	<5	<5	<5
9		x3	28	1+	<5	<5	<5	<5	<5	<5	<5	<5	<5
10	Fusarium sporotrichiodes	x1	16	-	<5	<5	<5	<5	<5	<5	<5	<5	<5
11		x2	16	1+	<5	**23**	<5	**3**	<5	<5	<5	<5	**121**
12		x3	16	2+	<5	**7**	<5	**3**	<5	<5	<5	<5	**13**
13		x1	20	-	<5	<5	<5	<5	<5	<5	<5	<5	<5
14		x2	20	1+	<5	**34**	<5	**5**	<5	<5	<5	**36**	**187**
15		x3	20	1+	**25**	**794**	**5?**	**15**	<5	<5	**30**	**853**	**6945**
16		x1	28	-	<5	<5	<5	**6**	<5	<5	<5	<5	<5
17		x2	28	3+	<5	**14**	<5	**4**	<5	<5	<5	**6**	**15**
18		x3	28	5+	<5	<5	<5	**6**	<5	<5	<5	**3**	**4**

Table 12. Mould growth and mycotoxin formation (*F. culmorum and F. poae from VTT*)

(Mycotoxin concentrations are expressed as µg/kg as is)

Sample No.	Micro-organism	Inoculum rate	Incubation Temp	Visual growth	DAS	Neosolaniol	Fusarenon X	DON	3-Ac DON	15-Ac DON	NIV	HT-2	T-2
19	Fusarium culmorum	x1	16	-	<5	<5	<5	<5	<5	<5	<5	<5	<5
20		x2	16	3+	<5	<5	<5	**357**	**149**	<5	<5	<5	<5
21		x3	16	5+	<5	<5	<5	**13**	**5**	<5	<5	<5	<5
22		x1	20	-	<5	<5	<5	4	<5	<5	<5	<5	<5
23		x2	20	-	<5	<5	<5	**361**	**428**	<5	<5	<5	<5
24		x3	20	3+	<5	<5	<5	**16**	<5	<5	<5	<5	<5
25		x1	28	1+	<5	<5	<5	**12**	<5	<5	<5	**19**	**16**
26		x2	28	1+	<5	<5	<5	**39**	**7**	<5	<5	<5	<5
27		x3	28	2+	<5	<5	<5	**14**	<5	<5	<5	<5	<5
28	Fusarium poae	x1	16	-	<5	<5	<5	**4**	<5	<5	**4**	<5	<5
29		x2	16	-	<5	<5	<5	**6**	<5	<5	<5	**13**	**7**
30		x3	16	-	<5	<5	<5	**22**	<5	<5	<5	<5	<5
31		x1	20	-	<5	<5	<5	**8**	<5	<5	<5	<5	<5
32		x2	20	-	<5	<5	<5	10	<5	**6**	**22**	<5	<5
33		x3	20	1+	<5	<5	<5	<5	<5	<5	<5	<5	<5
34		x1	28	-	<5	<5	<5	<5	<5	<5	<5	<5	<5
35		x2	28	-	<5	<5	<5	**5**	<5	<5	<5	**14**	<5
36		x3	28	5+	<5	<5	<5	<5	<5	<5	<5	<5	<5

Table 13. Mould growth and mycotoxin formation (*F. scirpi and F. crookwellense from CABI Biosciences)*)

(Mycotoxin concentrations are expressed as µg/kg as is)

Sample No.	Micro-organism	Inoculum rate	Incubation Temp	DAS	Neosolaniol	Fusarenon X	DON	3-Ac DON	15-Ac DON	NIV	HT-2	T-2
1	(A) Fusarium scirpi	x1	16	16	<5	<5	<5	<5	<5	<5	<5	<5
2		x2	16	1471	19	<5	5	<5	<5	12	<5	<5
3		x3	16	28	5	<5	<5	<5	<5	<5	<5	<5
4		x1	20	21	9	<5	5	<5	<5	<5	<5	<5
5		x2	20	729	14	<5	<5	<5	<5	19	<5	<5
6		x3	20	24	15	<5	6	<5	<5	<5	<5	<5
7		x1	28	<5	<5	<5	<5	<5	<5	<5	<5	<5
8		x2	28	17	<5	<5	<5	<5	<5	<5	<5	<5
9		x3	28	<5	<5	<5	7	<5	<5	<5	<5	<5
10	(B) Fusarium crookwellense	x1	16	26	9	<5	<5	<5	<5	<5	<5	<5
11		x2	16	48	6	189	10	<5	<5	69	<5	<5
12		x3	16	26	<5	<5	10	7	7	<5	<5	<5
13		x1	20	27	<5	<5	<5	<5	<5	<5	<5	<5
14		x2	20	47	<5	274	8	<5	<5	94	<5	<5
15		x3	20	32	<5	<5	<5	<5	<5	9	<5	<5
16		x1	28	<5	<5	<5	6	<5	<5	<5	<5	<5
17		x2	28	7	<5	<5	5	<5	<5	13	<5	<5
18		x3	28	<5	<5	<5	9	<5	<5	9	<5	<5

Table 14. Mould growth and mycotoxin formation (*P. verrucosum* and *P citrinin* from CABI Biosciences*)*

(Mycotoxin concentrations are expressed as µg/kg as is)

Sample No.	Micro-organism	Inoculum rate	Incubation Temp	DAS	NEO	Fusarenon X	DON	3-Ac DON	15-Ac DON	NIV	HT-2	T-2	Citrinin
19	(C) Penicillium citrinum	x1	16	<5	<5	<5	5	<5	<5	<5	<5	<5	18
20		x2	16	<5	<5	<5	11	<5	<5	<5	<5	<5	43
21		x3	16	<5	<5	<5	9	<5	<5	<5	<5	<5	52
22		x1	20	<5	<5	<5	<5	<5	<5	<5	<5	<5	15
23		x2	20	<5	<5	<5	<5	<5	<5	<5	31	12	33
24		x3	20	<5	<5	<5	7	<5	<5	<5	<5	<5	10
25		x1	28	<5	<5	<5	16	<5	<5	<5	<5	<5	16
26		x2	28	<5	<5	<5	<5	<5	<5	<5	<5	<5	100
27		x3	28	<5	<5	<5	7	<5	<5	<5	<5	<5	69
28	(D) Penicillium verrucosum	x1	16	<5	<5	<5	25	<5	<5	<5	<5	<5	4
29		x2	16	<5	<5	<5	5	<5	<5	<5	<5	<5	383
30		x3	16	<5	<5	<5	9	<5	<5	<5	<5	<5	29
31		x1	20	<5	<5	<5	<5	<5	<5	<5	<5	<5	3
32		x2	20	<5	<5	<5	19	<5	<5	<5	<5	<5	10
33		x3	20	<5	<5	<5	6	<5	<5	<5	<5	<5	2
34		x1	28	<5	<5	<5	7	<5	<5	<5	6	8	<0.1
35		x2	28	<5	<5	<5	11	<5	<5	<5	<5	<5	8588
36		x3	28	<5	<5	<5	14	<5	<5	<5	<5	<5	<0.1

4.3 Behaviour of mycotoxins during malting with inoculated barley

4.3.1 *F. sporotrichioides*

The inoculation with *F. sporotrichioides* (VTT reference number D-72014) was repeated on the pilot scale, using 50kg of barley and 10 litres mould culture, as described in the Methods section. A control was also carried out, in which barley was sprayed with clean water instead of mould culture. After 4 weeks incubation the barleys were malted and samples taken during processing for toxin analysis. However, the control batch was not kilned, thus toxin concentrations were only available for the steeping and germination stages.

Because of the high level of mycotoxins expected, process samples during malting were not analysed for routine malt quality parameters, since these analytical methods are difficult to conduct with the required level of bio-security. Toxin analysis at BRi is conducted in a custom-built laboratory with the appropriate health and safety facilities.

Mycotoxin data is shown in Table 15. Toxin concentrations are corrected for recovery and for sample moisture and expressed on a dry weight basis. Significant formation of DON and its acetylated derivatives, together with nivalenol (NIV) was observed in the control batch, presumably due to *Fusarium* species naturally present on the barley, but there was no formation of T-2 toxin, HT-2 toxin, DAS or NEO. Some FUS-X was detected in the barley after incubation, but this disappeared during steeping. The batch inoculated with *F. sporotrichioides* contained only traces of DON but significant quantities of neosolaniol, T-2 toxin and HT-2 toxin at the start of malting, as expected from the small scale trials.

During steeping the concentrations of T-2 toxin and neosolaniol fell substantially, probably due to extraction into the steep water. It is also possible that some of the T-2 was hydrolysed to HT-2, since concentrations of this toxin increased during steeping, although not to the same extent as the fall in T-2. There was some fluctuation in concentrations during germination, followed by a small increase during kilning, particularly for T-2.

No FUS-X or DAS was present in the barley at the start of malting, and none was formed during processing. Levels of DON and NIV were significantly lower in the inoculated batch than in the control batch, suggesting that the presence of large amounts of *F. sporotrichioides* inhibited the growth of other Fusarium species naturally present on the grain.

Overall changes during the malting process can be seen more easily when the concentrations are calculated per batch and expressed in terms of the dry weight of the starting barley (after mould inoculation and incubation). This is shown in Figure 4A.

Table 15. Development of T-2, HT-2 and neosolaniol during malting of barley inoculated with *F. sporotrichioides*

Sample	Inoculum	Mycotoxin concentration (µg/kg dry weight)								
		DAS	NEO	FUS-X	DON	3-Ac DON	15-Ac DON	NIV	HT-2	T-2
Barley	Water	<5	<5	127	3379	859	578	308	<5	<5
Casting		<5	<5	<5	2503	183	296	117	<5	<5
Germination Day 1		<5	<5	<5	3364	89	263	263	<5	<5
Germination Day 2		<5	<5	<5	2315	70	221	168	<5	<5
Germination Day 3		<5	<5	<5	2232	116	108	144	<5	<5
Germination Day 4		<5	<5	<5	1850	82	70	763	<5	<5
Barley	F. sporotrichioides	<5	276	<5	21	<5	<5	<5	241	2083
Casting		<5	192	<5	33	<5	<5	38	702	1165
Germination Day 1		<5	31	<5	86	88	31	13	629	697
Germination Day 2		<5	69	<5	108	NA	17	101	724	517
Germination Day 3		<5	19	<5	73	60	11	NA	375	576
Germination Day 4		<5	60	<5	61	23	<5	70	629	856
Kiln Top		<5	151	<5	90	28	9	22	530	1061
Kiln Middle		<5	151	<5	50	22	5	15	770	1079
Kiln Bottom		<5	152	<5	98	13	31	6	804	1189
Rootlets		<5	448	<5	179	5	7	6	36	37
Derooted Kilned malt bulk		<5	120	<5	109	<5	<5	<5	741	902

Figure 4A. Mass balance for T-2 and HT-2 toxins during malting of barley inoculated with *F sporotrichioides*

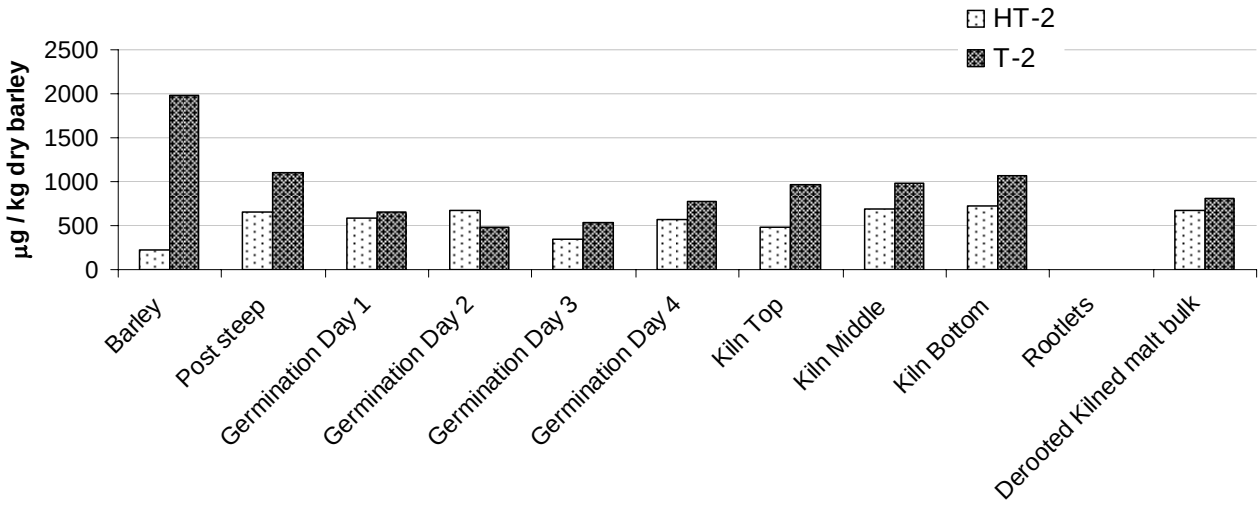
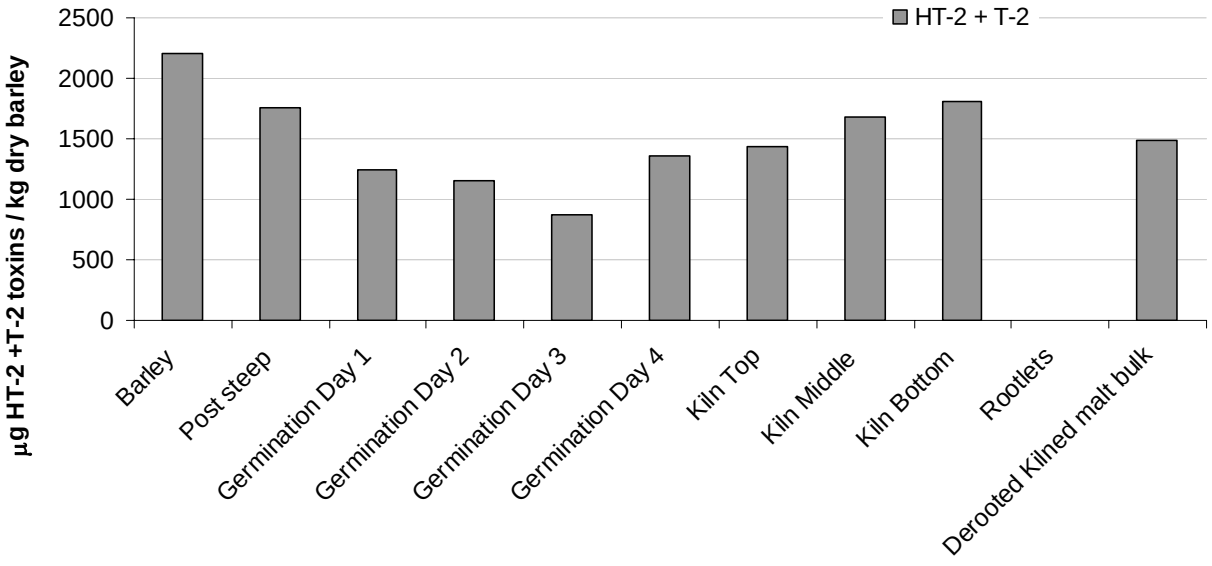


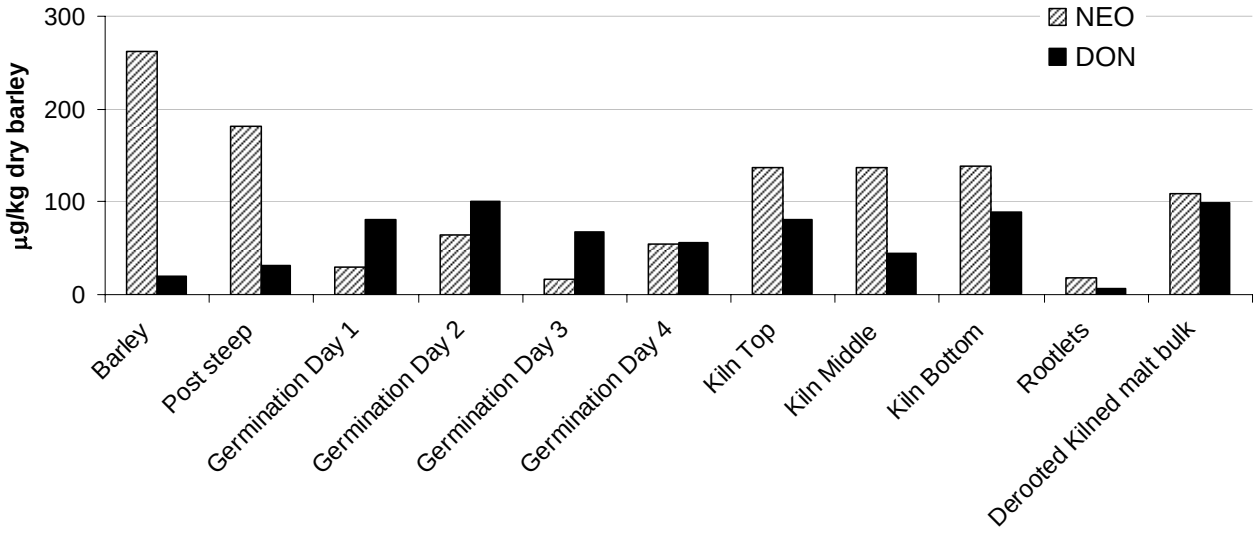
Figure 4A shows clearly the concentration fall in the concentration of T-2 toxin during steeping, accompanied by a much smaller increase in levels of HT-2. During germination concentrations of both toxins fluctuate slightly, followed by a small increase during kilning. However, it is clear from Figure 4B that overall there is a significant loss in the combined concentration of these toxins during malting. Losses to the rootlets are negligible.

Figure 4B. Mass balance for the sum of HT-2 and T-2 toxins during malting of barley inoculated with *F. sporotrichioides*



Neosolaniol (Table 15 and Figure 5) also exhibited a significant fall in concentration during steeping, followed by further substantial reductions during germination. There was some synthesis during kilning, but the final malt contained less than half that originally present in the barley. Unlike T-2 and HT-2 toxins, some neosolaniol was found in the rootlets.

Figure 5. Mass balance for Neosolaniol and DON during malting of barley inoculated with *F. sporotrichioides*



If the pattern of behaviour for T-2, HT-2 and neosolaniol is compared with that of DON (Figure 5), it is evident that losses during steeping are substantially higher than those for DON, while formation during malting is relatively small. With DON, there is obviously some potential for a net increase during malting.

4.3.2 *F. crookwellense*

In this study, samples were taken during the incubation period following inoculation with *F. crookwellense* culture in order to compare mycotoxin development on the pilot scale (50kg) with the small (250g) scale. Results are shown in Figure 6. As expected, fusarenon-X was the main toxin present, but significant development of nivalenol was also observed, together with some DON. There was a pronounced lag phase before the appearance of DON and NIV compared with that of fusarenon-X.

Traces of acetylated DON (15-aecty DON) were also detected.

The behaviour of these mycotoxins during the pilot malting stage is shown in Table 16 and Figure 7 (A and B).

Figure 6. Development of toxins during incubation of barley inoculated with *F. crookwellense* (pilot scale)

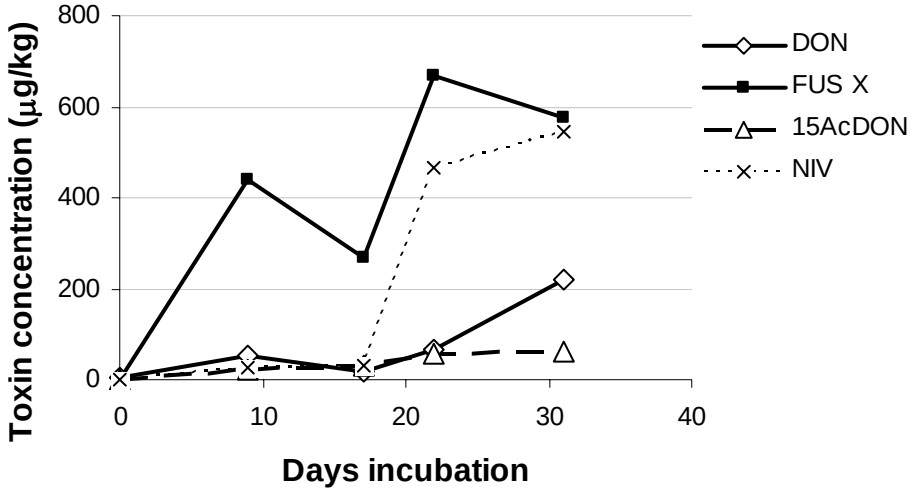
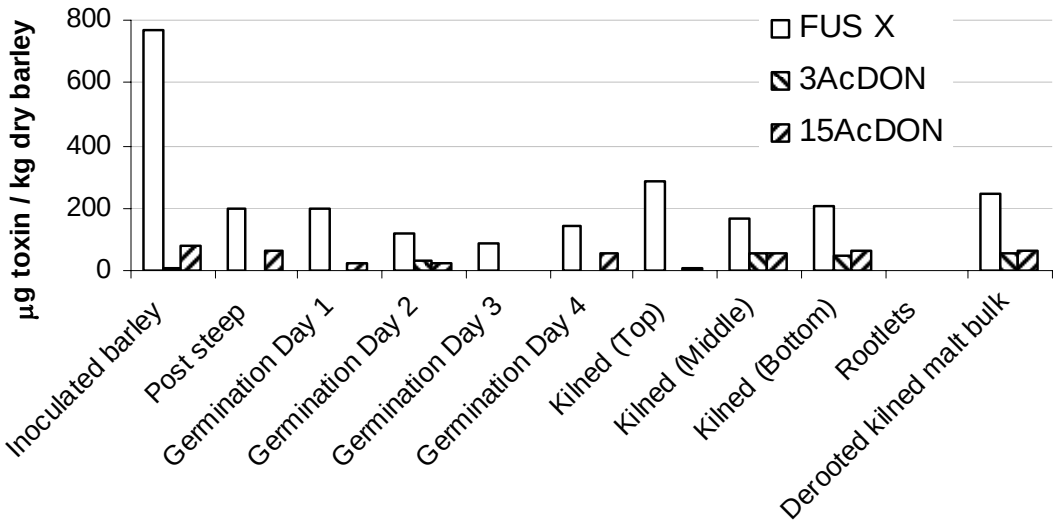


Table 16. Behaviour of fusarenon-X during malting of barley inoculated with *F. crookwellense*

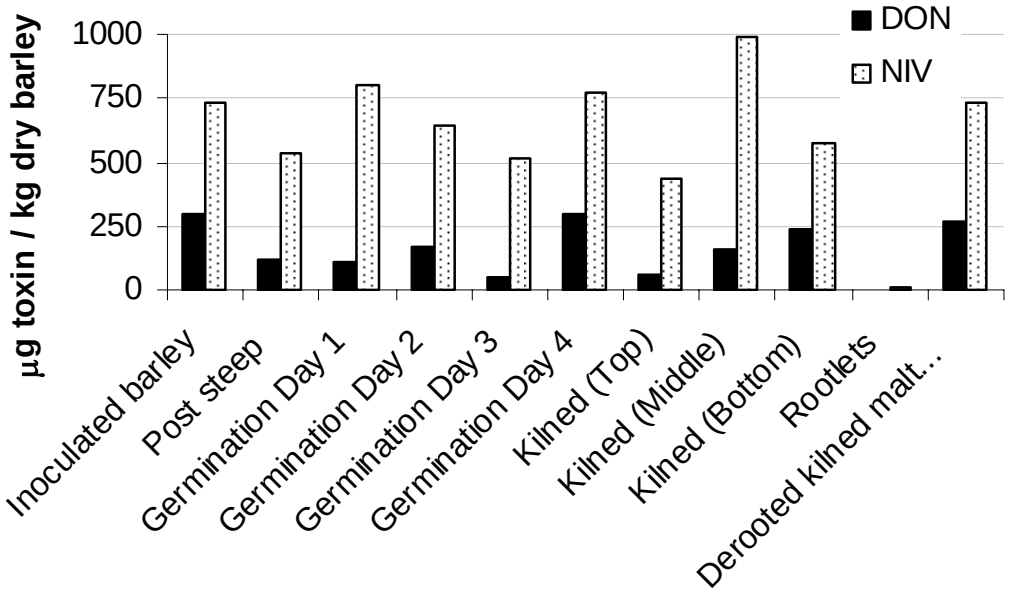
Sample	µg toxin / kg (dry weight)				
	FUS-X	3-AC DON	15-Ac DON	DON	NIV
Barley (after inoculation / incubation)	768	5	83	293	729
Casting	202	<5	64	118	538
Germination Day 1	200	<5	25	113	821
Germination Day 2	126	32	21	172	663
Germination Day 3	88	<5	<5	49	533
Germination Day 4	150	<5	59	310	814
Kiln Top	303	<5	8	59	461
Kiln Middle	178	57	57	172	1056
Kiln Bottom	216	49	71	254	615
Rootlets	70	<5	6	128	748
Derooted Kilned malt bulk	260	56	68	288	781

Figure 7A. Mass balance for Fusarenon-X during malting of barley inoculated with *F. crookwellense*



Significant quantities of the fusarenon-X present in the barley were washed out during the steeping stage, and the proportion lost was higher than that observed with most other trichothecenes investigated in this project, Although there was some fluctuation in measured concentrations during germination (probably due to sampling), levels of fusarenon-X in the final malt bulk were close to those in the steeped grain. Traces of acetylated DON species were also detected. The 3-acetyl DON appeared mainly during the kilning stages, while 15-acetylated DON was present at similar concentrations in the starting barley and in the kilned malt.

Figure 7 B. Mass balance for DON and NIV during malting of barley inoculated with *F. crookwellense*

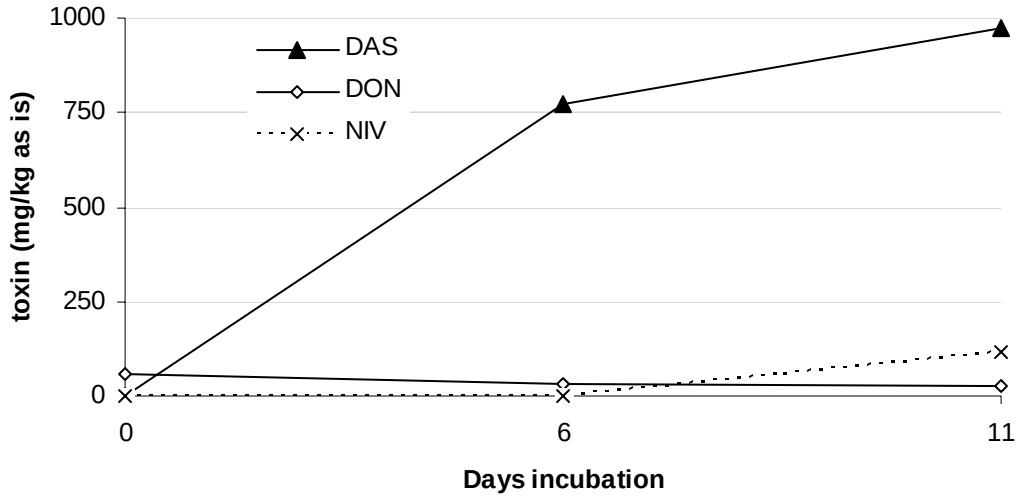


As already noted, significant quantities of NIV and some DON were also formed during the incubation with *F. crookwellense* (Figure 6). Analysis of the processing samples suggested that these two toxins behaved similarly during malting. Losses during steeping were relatively small, confirming the observations with the *F. sporotrichioides* trial, but there were indications of further toxin synthesis during germination and kilning, so that the finished malt bulk contained similar concentrations of both DON and NIV to the starting barley.

4.3.3 *F. scirpi*

As with the small scale inoculations, the pilot scale incubation of barley with *F. scirpi* produced substantial quantities of DAS, with small amounts of DON and NIV but no other trichothecenes (Figure 8).

Figure 8. Development of toxins during incubation of barley inoculated with *F. scirpi* (pilot scale)



Toxin concentrations during malting are shown in Table 17. During steeping a substantial proportion of this pre-formed DAS was washed out. The concentrations of DON and NIV also fell during steeping, but not to the same extent as DAS, confirming the previous observations with the *F. crookwellense* trial. Apart from some minor fluctuations, probably due to sampling, DAS concentrations continued to fall during germination. There was no evidence of renewed synthesis of DAS during malting and the finished malt contained around 5% of the DAS originally present in the barley after incubation.

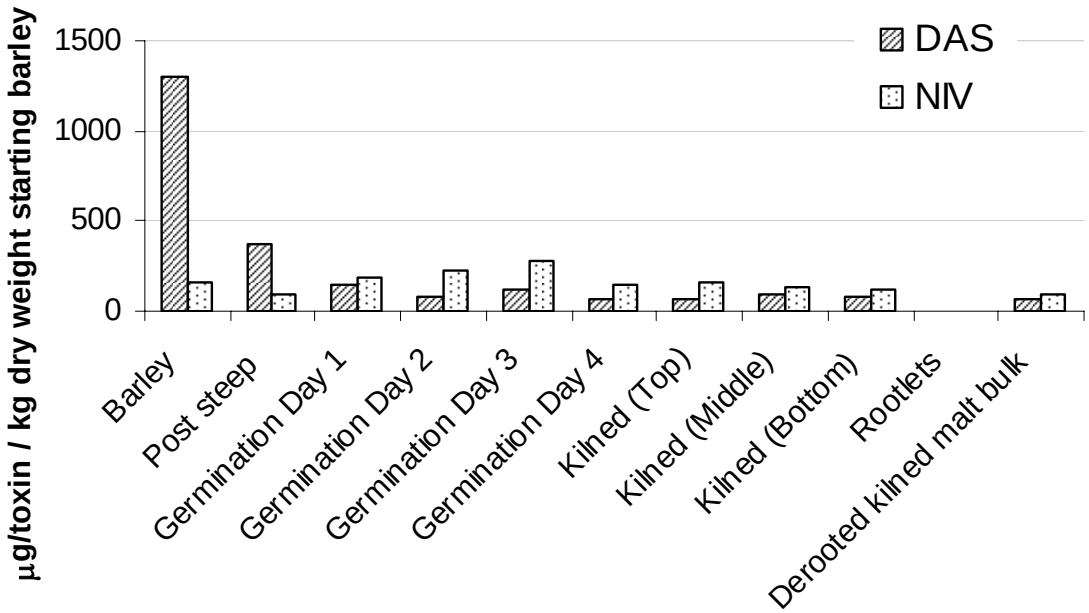
NIV, on the other hand, increased slightly during malting, and concentrations in the final malt were only slightly less than those in the starting barley. This pattern was also observed with the *F. crookwellense* infected batch (Figure 7B).

Table 17. Behaviour of DAS during malting of barley inoculated with *F. scirpi*

Sample	µg toxin / kg (dry weight)		
	DAS	DON	NIV
Barley (after incubation)	1295	67	156
Casting	375	42	91
Germination Day 1	151	39	185
Germination Day 2	88	13	240
Germination Day 3	126	20	298
Germination Day 4	74	9	149
Kiln Top	76	26	174
Kiln Middle	96	16	137
Kiln Bottom	92	18	124
Rootlets	6	<5	105
Derooted Kilned malt bulk	69	11	107

The concentration of DAS and NIV, expressed in terms of the original dry weight of the barley at the start of malting, are shown in Figure 9.

Figure 9. Mass balance for DAS and NIV during malting of barley inoculated with *F. scirpi*



4.3.4 *Penicillium verrucosum*

Unlike the small scale inoculations, barley inoculated and incubated with *Penicillium verrucosum* on the pilot scale also exhibited some growth of *Fusarium* moulds and significant development of some trichothecenes was observed in addition to citrinin and ochratoxin A. This batch was processed one month prior to the *F. crookwellense* batch, so there is no possibility of contamination of the *P. verrucosum* batch with *F. crookwellense.* Since *P. verrucosum* is not known to produce trichothecenes, it seems probable that the barley was naturally contaminated with a *Fusarium* species which did not compete with the applied *Penicillium* inoculum and could thus flourish under the high moisture conditions of the incubation, at least on the pilot scale.

Changes in the concentrations of citrinin and ochratoxin A in samples taken during processing are shown in Table 18. Although the very low concentration of citrinin immediately after steeping might indicate potential leaching by steep water, the high values during the rest of germination suggest that the low value is more likely to be a sampling anomaly. However, the relatively low concentration in the finished bulk suggests that there are significant losses of citrinin during kilning.

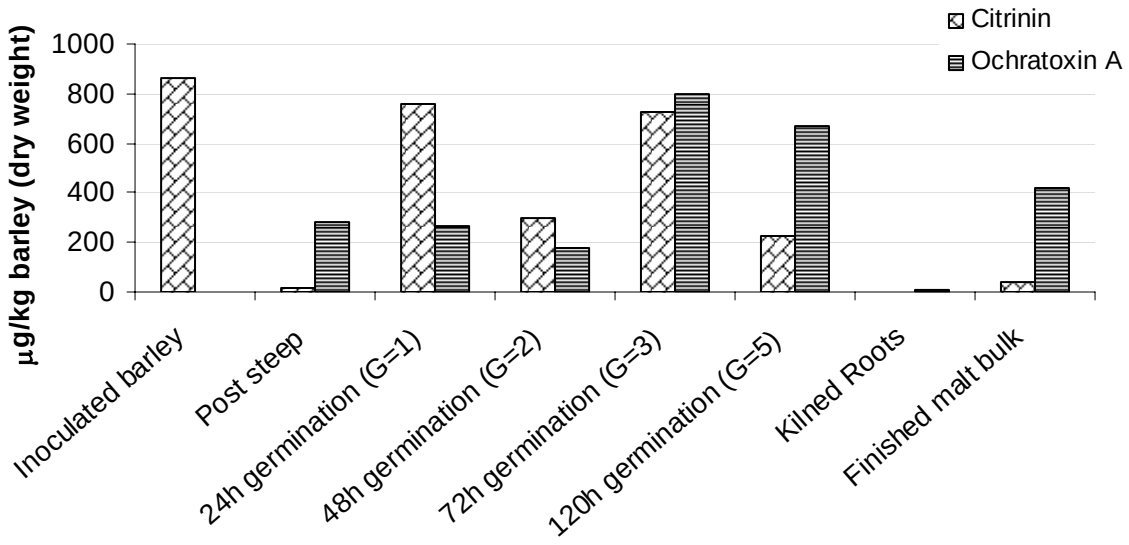
Ochratoxin concentrations showed little correspondence with those of citrinin, and there was evidence of OA formation during the later stages of germination and early kilning.

Table 18. Citrinin and Ochratoxin A development during malting of barley inoculated with *P. verrucosum*

	µg toxin / kg (dry)	
Sample	**Citrinin**	**OA**
Barley (after inoculation and incubation)	885	NA
Casting	16	294
Germination (Day 1)	803	287
Germination (Day 2)	327	194
Germination (Day 3)	814	889
Germination (Day 5)	265	791
Kilned Roots	48	467
Finished malt bulk	56	633

A mass balance for citrinin and ochratoxin A, in terms of the dry weight of the starting barley, is presented in Figure 10. This illustrates more clearly an overall trend for citrinin to fall during malting and particularly during kilning, whereas ochratoxin A tends to increase during processing if viable mould is present.

Figure 10. Mass balance for citrinin and ochratoxin A during malting of barley inoculated with *P. verrucosum*



Concentrations of trichothecenes during malting are shown in Table 19. DON, acetylated DON species, nivalenol and fusarenon-X were all detected, but the patterns differed between the individual mycotoxins. Fusarenon-X was present at a relatively high concentration in the barley at the end of the inoculation and incubation stage, prior to the commencement of malting. Significant quantities of this mycotoxin were leached out during steeping, as was observed with the *F. crookwellense* study, and there were further losses during kilning. The final malt bulk contained less than half of the fusarenon-X originally present in the barley at the start of malting.

Table 19. Development of Trichothecenes during malting of barley inoculated with *P. verrucosum*

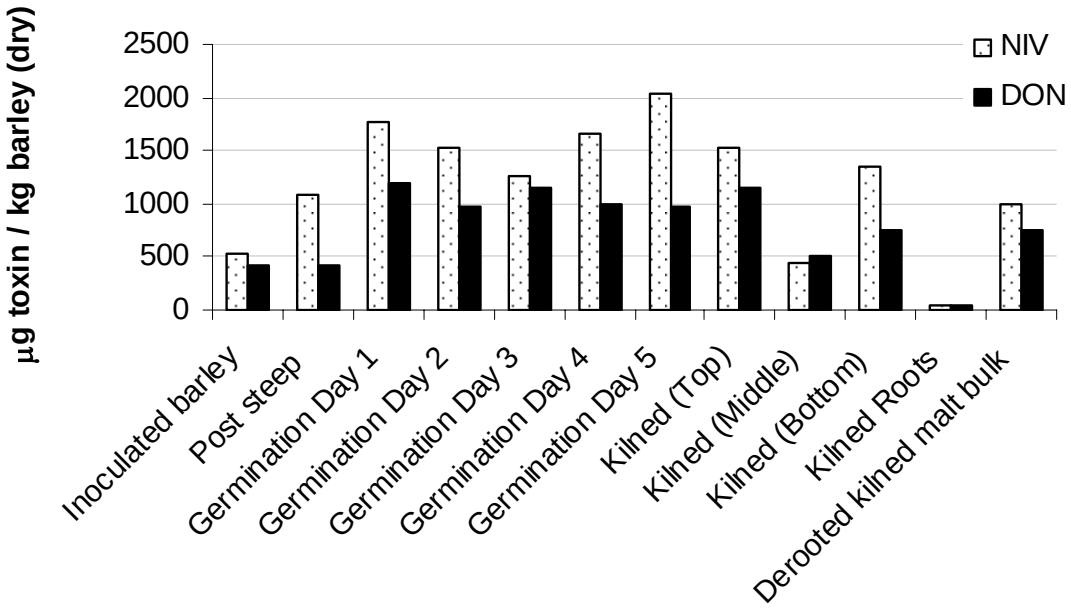
	µg /kg (dry)				
Sample	**FUS X**	**DON**	**3AcDON**	**15AcDON**	**NIV**
Barley (after inoculation and incubation)	452	434	23	83	544
Casting	233	446	31	28	1123
Germination Day 1	227	605	43	29	1539
Germination Day 2	169	1356	104	81	1706
Germination Day 3	100	1275	90	60	1216
Germination Day 4	261	1018	151	181	1498
Germination Day 5	242	1088	218	33	2225
Kilned (Top)	216	1735	100	88	2298
Kilned (Middle)	84	763	96	91	684
Kilned (Bottom)	273	1155	132	101	2054
Kilned Roots	114	2768	139	92	3302
Finished malt bulk	176	1144	88	81	1506

In contrast, DON and nivalenol were not leached out to any extent during steeping, as was observed in the previous studies, and concentrations increased significantly during germination. There were some losses of both mycotoxins during kilning, but the concentration in the finished malt remained significantly higher than in the starting barley.

There was also a significant increase of 3-acetyl DON during germination. About half of this was lost during kilning, but the final malt contained significantly more than the starting barley. With 15-acetyl-DON, although there were some variations between samples, there was little overall change between the starting barley and the final malt. This pattern was also observed in the *F. crookwellense* study.

A mass balance for DON and nivalenol, in terms of the dry weight of the starting barley, is presented in Figure 11.

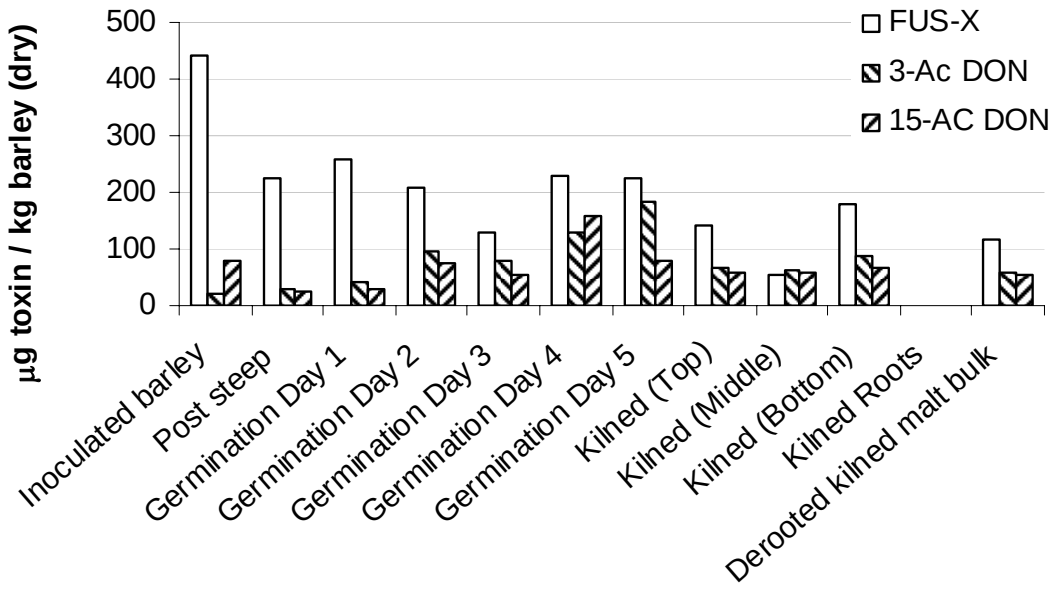
Figure 11. Mass balance for DON and Nivalenol during malting of barley inoculated with *P. verrucosum*



The overall trends are similar to those observed for DON and nivalenol during malting with barleys inoculated with the various *Fusarium* moulds although the relative proportions of toxins are quite different.

A mass balance for Fusarenon-X and the acetylated DON species is shown in Figure 12, and confirms the trends for these toxins observed during the *F. crookwellense* study.

Figure 12. Mass balance for Fusarenon-X and the acetylated DON species during malting of barley inoculated with *P. verrucosum*



4.4. Brewing with artificially infected malts

4.4.1 Wort and beer quality

Malts prepared from barleys inoculated with the various moulds were brewed in the BRI pilot brewery in order to investigate potential carryover of mycotoxins from barley into beer. In view of the high levels of associated mould, it is not surprising that brewing performance was affected in most cases (see Table 20). The most pronounced effect was the very poor wort clarity, which was apparent with all four moulds.

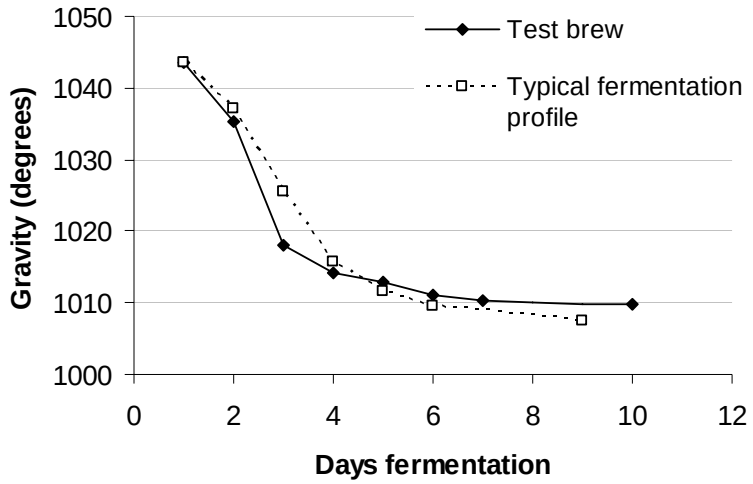
Table 20. Brewing and fermentation performance for malts prepared from artificially infected barleys

	Brew A ***F. sporotrichioides***	***Brew B*** ***F. crookwellense***	***Brew C*** ***F. scirpi***	***Brew D*** ***P. verrucosum***
Lautering performance	Raking needed	Satisfactory	Satisfactory	Raking needed
Wort clarity	Very poor	Very poor	Very poor	Very poor
Extract yield (litre °Pl at fermentat^{n} gravity)	948	957	975	923
Fermentation performance	Slightly high final gravity	Satisfactory	Slightly high final gravity	Satisfactory
Yeast viability	89	92	92	89

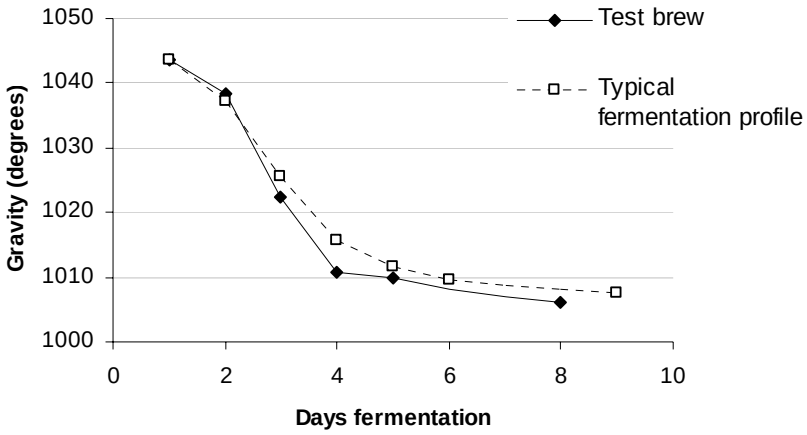
Fermentation profiles for each brew are shown in Figure 13. These indicate that although the final gravities achieved with *F. sporotrichioides* and *F. scirpi* were slightly higher than typical profiles with the same yeasts, the effects on fermentation performance were in generally insignificant. Yeast viability was also unaffected. Analysis of the four worts and beers for standard quality parameters is shown in Tables 21 and 22.

Figure 13. Fermentation profiles

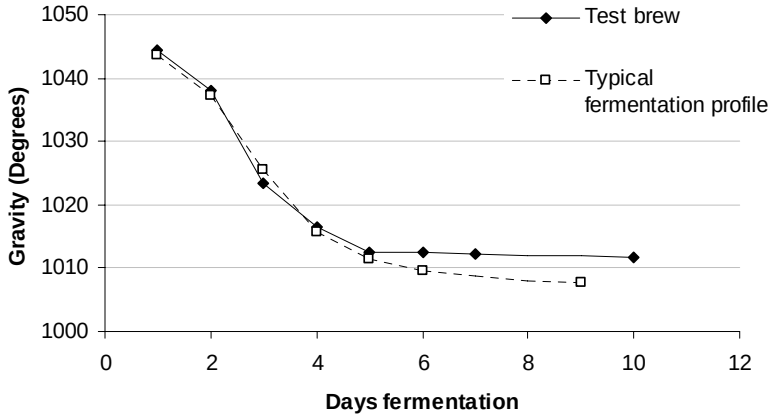
A. *F. sporotrichioides*



B. *F. crookwellense*



C. *F. scirpi*



D. *P. verrucosum*

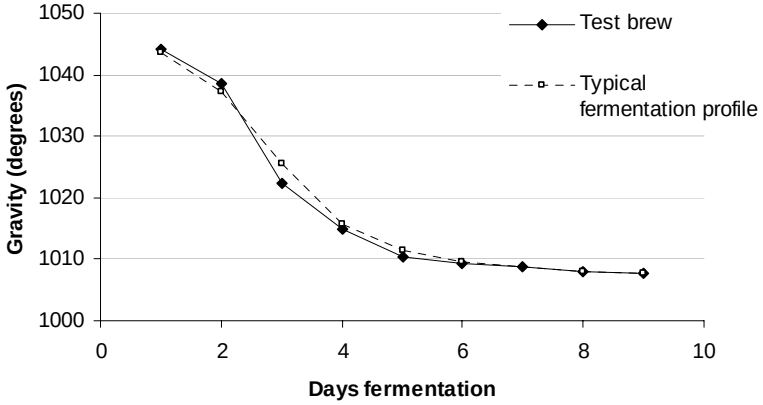


Table 21. Wort analyses

	Brew A F. sporotrichioides	***Brew B F. crookwellense***	***Brew C F. scirpi***	***Brew D P. verrucosum***	**Typical values**
pH	5.31	5.47	5.43	5.39	5.56
Colour (EBC)	35	22	31	50	7.5
Fermentability (%)	70	72	69	67	73
Free Amino N (mg/litre)	182	262	197	242	140 - 170
Total soluble N (mg/litre)	938	1232	929	1201	760 - 900
Bitterness (BU)	32	28	34	35	33-38

Table 22 . Beer analyses

Parameter	***Brew A F. sporotrichioides***	***Brew B F. crookwellense***	***Brew C F. scirpi***	***Brew D P. verrucosum***	**Typical values**
pH	4.00	4.17	4.02	4.36	3.9 – 4.3
Colour (EBC)	6.7	9.1	6.3	11	5.1 – 5.6
Present gravity	8.92	5.60	8.97	7.34	4.6 – 6.4
Attenuation limit	6.40	5.42	6.83	7.69	5.0 – 5.4
Bitterness (BU)	17	17	21	18	22 - 23
Free Amino N (mg/L)	47.6	101	47.4	122	28 - 45
Total Soluble N (mg/L)	628	691	559	662	450 - 600
Haze (EBC)	0.55	0.58	0.64	1.69	0.2 – 0.3
Foam stability at 30 mins (secs)	231	221	247	211	248 -286
Ethanol (% v/v)	4.46	4.81	4.62	4.66	4.9 – 5.1
Acetaldehyde (mg/L)	5.2	2.7	8.1	1.4	2.9 – 9.5
Dimethyl sulphide (µg/L)	17	23	17	20	21
Diacetyl (mg/L)	0.07	0.10	0.10	0.08	0.05 – 0.1
Ethyl acetate (mg/L)	12.1	11.7	13.8	8.3	15 - 25
***iso*-butyl acetate (mg/L)**	<0.06	<0.06	<0.06	<0.06	<0.06
***iso*-amyl acetate (mg/L)**	0.7	0.59	0.97	0.43	1 - 2
Ethyl hexanoate (mg/L)	0.09	0.08	0.1	0.05	0.14
***n*-propanol (mg/L)**	16.9	15.2	14.3	11.8	10 - 11
***iso*-butanol (mg/L)**	20.8	18.9	20.8	17.1	13 - 17
***iso*-amyl alcohol (mg/L)**	74.7	90.2	68.1	73.1	71 - 77
Gushing	+++	+	++	++	none

The mould-infected malts all gave much darker worts than normal, and some of this colour carried through to the beers. Levels of free amino and total soluble nitrogen were also significantly higher than normal in both the worts and the beers. Flavour volatiles were relatively unaffected, although there was some tendency for the infected beers to contain slightly lower levels of esters and more higher alcohols. These effects, however, were not substantial. All the infected beers were significantly hazier than control beers and, as might be expected, they displayed a greater tendency to gush.

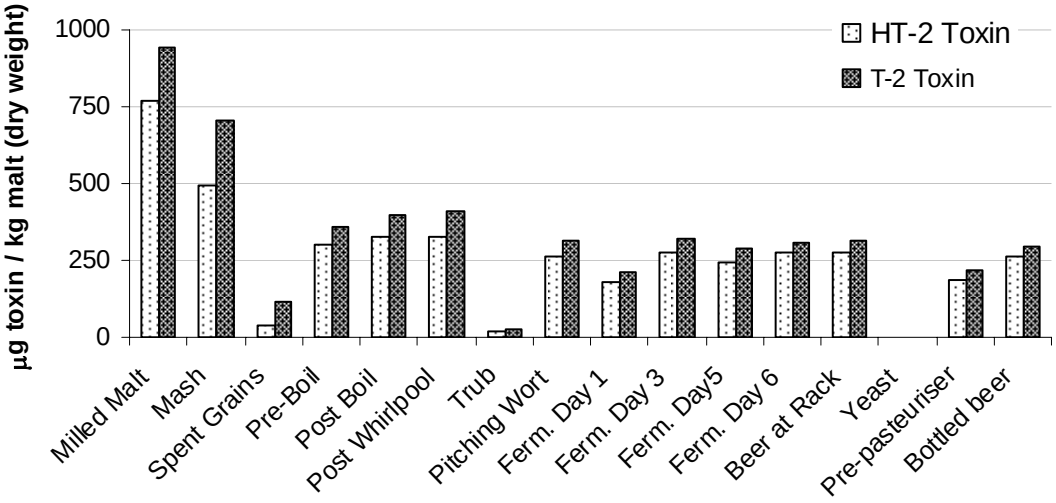
4.4.2 Survival of mycotoxins

4.4.2.1 HT-2 and T-2 (Brew A)

Malt prepared from barley inoculated with *F. sporotrichioides* was brewed in the BRI pilot brewery in order to investigate the carryover of HT-2 and T-2 toxins from malt into beer.

A mass balance for these toxins in brewing is illustrated in Figure 14. Toxin concentrations are expressed per kg dry weight of the starting malt.

Figure 14. Mass balance for T-2 and HT-2 during brewing (Brew A)



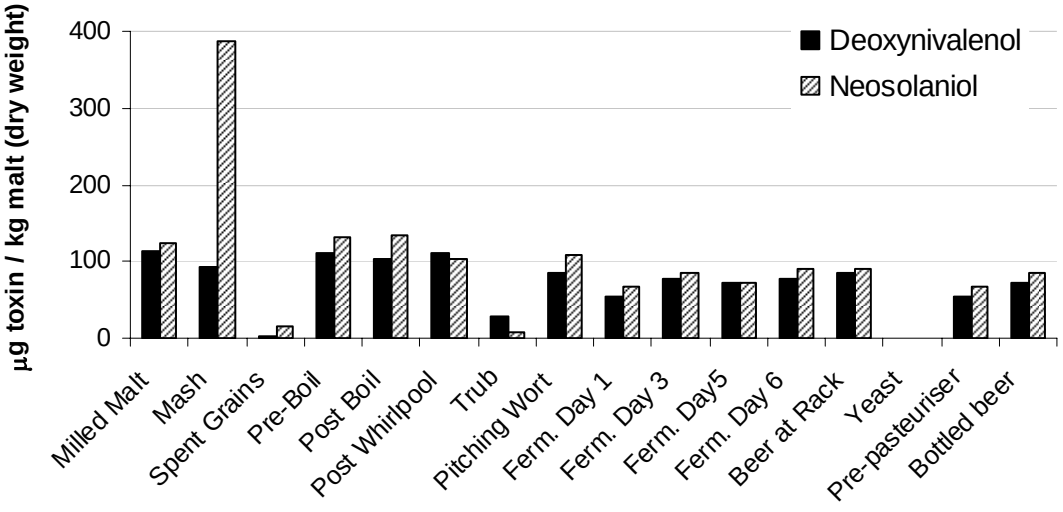
The data suggest that T-2 and HT-2 behave similarly during brewing. There is a significant drop in the concentration of both toxins during the mashing and lautering stages, a fall which exceeds the amount lost in the spent grains, suggesting that there may be some degradation, possibly enzymatic. However there is no further change during the boil, indicating that both T-2 and HT-2 are stable to heat. There are further small losses of both toxins with the trub (the coagulated proteinaceous and hop material which precipitates during the boil). Concentrations of both toxins remained constant during fermentation and pasteurisation. Levels in the final packaged beer were similar to those in the fermenting wort. Overall, around two thirds of the T-2 and HT-2 present in the starting malt was lost during brewing.

The actual concentrations of T-2, HT-2 and other trichothecene mycotoxins in each fraction are shown in Table 23.

4.4.2.2 Neosolaniol and DON (Brew A)

Malt prepared from barley inoculated with *F. sporotrichioides* was also used to follow the behaviour of NEO during brewing, since this toxin was also present in significant quantities. A mass balance for NEO is presented in Figure 15, together with results for DON for comparison.

Figure 15. Mass balance for Neosolaniol and DON during brewing (Brew A).



Apart from the very high level of NEO in the mash (which appears to be an anomalous result), Figure 15 shows that the behaviour of NEO and DON is similar during brewing, and both show some differences to T-2 and HT-2. Unlike T-2 and HT-2, there is little loss during mashing and lautering. Both DON and NEO are stable during the boil. There are small losses of both toxins during fermentation, but the finished beer contains around two thirds of the NEO and DON present in the malt.

Acetylated DON species, FUS-X, DAS and NIV were not present in the starting malt and were not detected during the brewing process.

Table 23. Mycotoxins in brewing process fractions using malt prepared from barley inoculated with *F. sporotrichioides* (Brew A).

Mycotoxin	µg toxin / kg (litre) as is							
	Milled Malt	Mash	Spent Grains	Pre-Boil	Post Boil	Post Whirlpool	Trub	Pitching Wort
Deoxynivalenol	109	22	2.5	17	17	18	75	14
Neosolaniol	120	93	12	20	22	17	19	18
HT-2 Toxin	741	119	26	46	53	54	52	43
T-2 Toxin	902	169	82	54	65	67	63	52
Fusarenon-X	<5	<5	<5	<5	<5	<5	<5	<5
3-Ac DON	<5	<5	<5	<5	<5	<5	<5	<5
15-Ac DON	<5	<5	<5	<5	<5	<5	<5	<5
Nivalenol	<5	<5	<5	<5	<5	<5	<5	<5
DAS	<5	<5	<5	<5	<5	<5	<5	<5

Mycotoxin	µg toxin / kg (litre) as is							
	Ferm. Day 1	Ferm. Day 3	Ferm. Day 5	Ferm. Day 6	Beer at Rack	Yeast	Pre-pasteuriser	Bottled beer
Deoxynivalenol	9	13	12	13	14	2.5	9	12
Neosolaniol	11	14	12	15	15	2.5	11	14
HT-2 Toxin	30	46	40	46	46	2.5	31	43
T-2 Toxin	35	53	48	51	52	2.5	36	49
Fusarenon-X	<5	<5	<5	2.5	2.5	2.5	2.5	2.5
3-Ac DON	<5	<5	<5	2.5	2.5	2.5	2.5	2.5
15-Ac DON	<5	<5	<5	2.5	2.5	2.5	2.5	2.5
Nivalenol	<5	<5	<5	2.5	2.5	2.5	2.5	2.5
DAS	<5	<5	<5	2.5	2.5	2.5	2.5	2.5

4.4.2.3 Fusarenon-X (Brew B)

The malt prepared with *F. crookwellense* was also brewed and the carry-over of FUS-X and NIV were followed through the brewing process. A mass balance for FUS-X is shown in Figure 16A. Because of the difference in concentration between FUS-X and NIV, the mass balance for NIV is shown separately in Figure 16B.

Figure 16A. Mass balance for Fusarenon-X during brewing (Brew B).

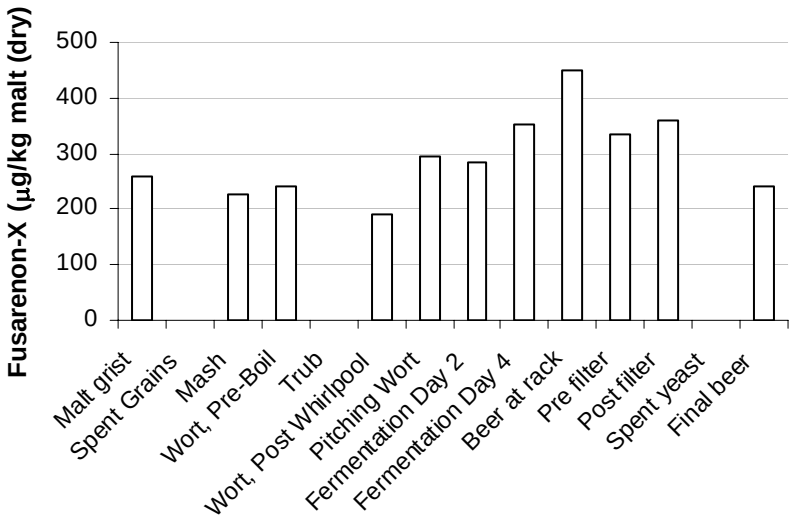
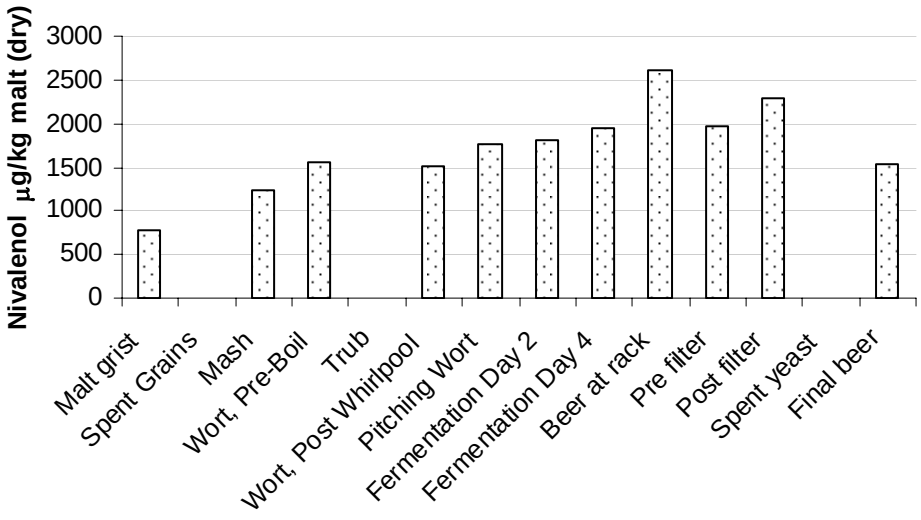


Figure 16 B. Mass balance for Nivalenol during brewing (Brew B).



It can be seen from Figure 16 that both FUS-X and NIV survived processing and were transferred almost quantitatively into the beer. Losses in to the spent grains, trub and yeast were negligible. A variation in concentrations and apparent increase during fermentation was observed for each mycotoxin – this is likely to be due to the heterogeneity of these samples and the presence of entrained yeast. It is interesting to note that more NIV was detected in the mash and wort samples than was found in the original malt. This was also observed for

NIV in Brew D and may be due to the increased ease of extraction in the liquid mash compared with the solid malt.

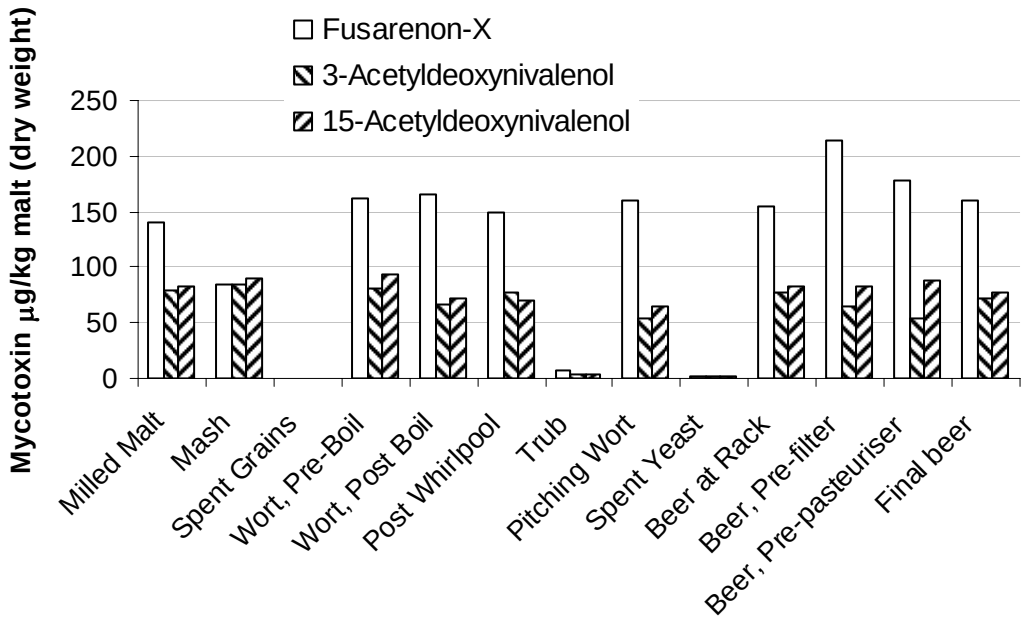
Actual concentrations of mycotoxins are shown in Table 24.

Table 24. Mycotoxins in brewing process fractions using malt prepared from barley inoculated with *F. crookwellense* (Brew B).

Fraction	Mycotoxin µg/kg (litre)	
	FUS-X	**NIV**
Milled malt grist	260	781
Mash	228	1243
Spent grains	0	0
Wort, pre-boil	241	1555
Wort, post whirlpool	190	1500
Trub	0	0
Pitching wort	296	1773
Fermentation, Day 2	284	1810
Fermentation, Day 4	353	1937
Beer at rack	448	2618
Spent yeast	0	0
Beer, pre-filter	334	1974
Beer, post filter	360	2296
Packaged, pasteurised beer	240	1546

As described in section 4.3.4, barley inoculated with *P. verrucosum* also developed FUS-X and acetylated DON species, significant quantities of which survived through into the malt, and therefore this process samples from this brew (Brew D) were also used to monitor the survival of FUS-X. A mass balance for FUS-X, 3-acetyl DON and 15-acetyl DON is shown in Figure 17, with results for DON and nivalenol in the same brew shown in Figure 18 for comparison. This brew was not sampled during fermentation; instead the filtered beer was sampled prior to pasteurisation to check if this had any effect on toxin levels.

Figure 17. Mass balance for Fusarenon-X, 3-acetyl DON and 15-acetyl DON *(P. verrucosum) (Brew D)*



As with Brew B, FUS-X survived well during brewing, with almost quantitative transfer from the malt grist to the final beer. Losses in the spent grains, trub and spent yeast were negligible, as were losses during filtration. The behaviour of DON and the acetylated DON species was very similar, with even lower losses of DON than in the *F. sporotrichioides* brew (Brew A).

NIV concentrations displayed greater fluctuations between the different sample points than did the other toxins. Most noticeably, concentrations of NIV in the mash and subsequent brewing fractions exceeded those in the malt. Since there is no possibility that mould could actually grow and generate mycotoxins under mashing conditions, this may be due to the poorer recovery of NIV during analysis, which, since all results are corrected for recovery, would magnify any variations due to sampling. It is also possible that the toxins could be more easily extracted from the liquid mash. It is clear, however, that a large proportion of the NIV survives the brewing process and persists into the beer.

Actual concentrations of mycotoxins in process fractions from Brew D are given in Table 25.

Figure 18. Mass balance for DON and NIV *(P. verrucosum)* (Brew D)

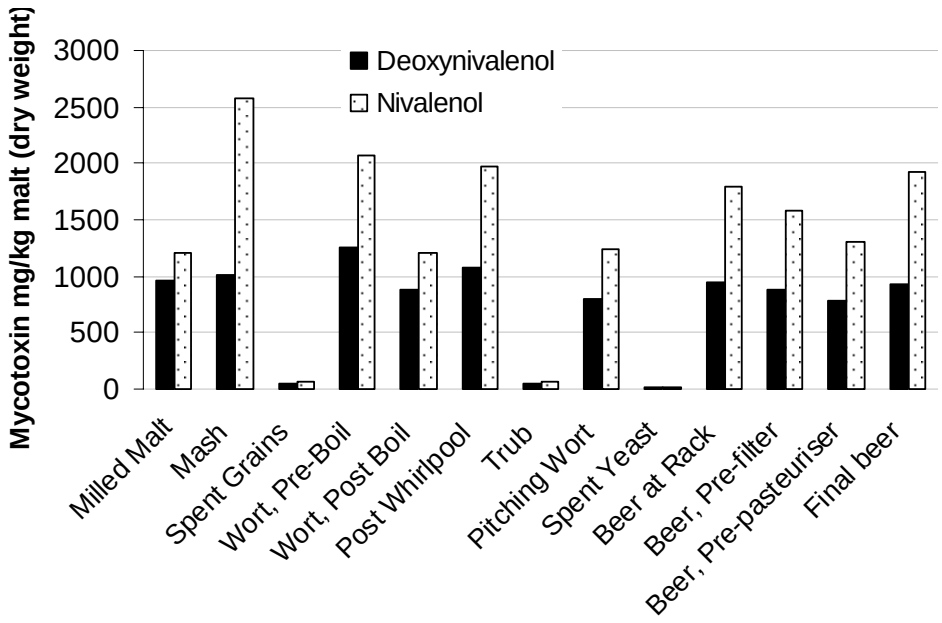


Table 25. Trichothecenes in brewing process fractions using malt prepared from barley inoculated with *P. verrucosum* (Brew D).

Fraction	Mycotoxin µg/kg (litre)				
	FUS-X	**3-Ac DON**	**15-Ac DON**	**DON**	**NIV**
Milled malt grist	132	74	78	908	1139
Mash	20	20	21	237	607
Spent grains	<5	<5	<5	31	46
Wort, pre-boil	24	12	14	186	308
Wort, post-boil	30	12	13	159	219
Trub	22	12	12	129	198
Wort, post whirlpool	27	14	13	196	358
Pitching wort	27	9	11	136	210
Beer at rack	26	13	14	161	304
Spent yeast	32	5	6	202	342
Beer, pre-filter	36	11	14	148	266
Beer, post filter	30	9	15	133	219
Packaged, pasteurised beer	27	12	13	158	325

4.4.2.4 DAS (Brew C)

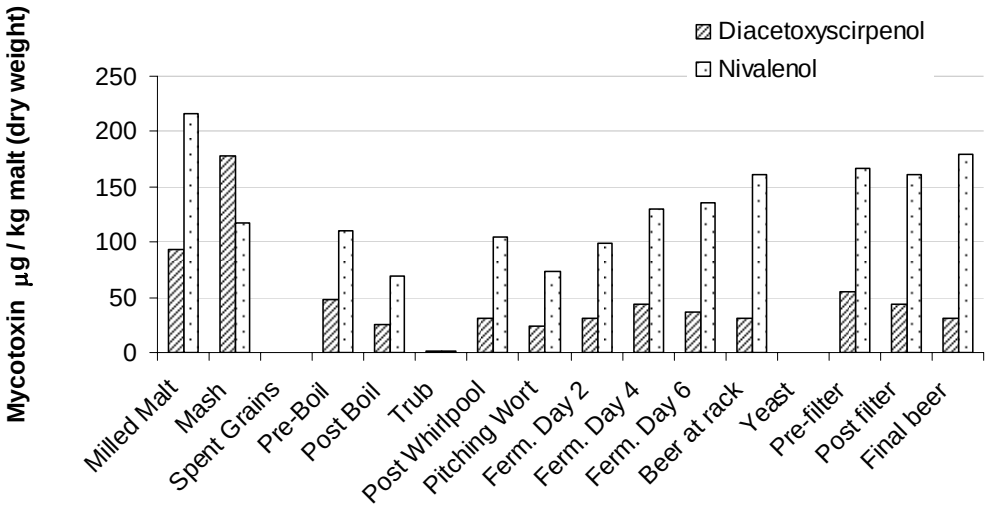
The malt prepared from barley inoculated with *F. scirpi* was brewed in the BRi pilot brewery in order to observe the potential for carry-over of DAS from malt into beer (Brew C). Since the malt contained significant quantities of nivalenol, this toxin was also monitored. As before, samples were taken throughout the brewing process for analysis of toxins. Concentrations of NIV and DAS in process fractions are shown in Table 26.

Table 26. DAS and NIV in brewing process fraction using malt prepared from barley inoculated with *F. scirpi* (Brew C).

Fraction	Mycotoxin µg/kg (litre)	
	DAS	**NIV**
Milled malt grist	88	205
Mash	43	28
Spent grains	<5	<5
Wort, pre-boil	7	16
Wort, post-boil	4	11
Trub	2	6
Wort, post whirlpool	5	17
Pitching wort	4	12
Fermentation, Day 2	5	16
Fermentation, Day 4	7	21
Fermentation. Day 6	6	22
Beer at rack	5	26
Spent yeast	<5	<5
Beer, pre-filter	9	27
Beer, post filter	7	26
Packaged, pasteurised beer	5	29

A mass balance for DAS and nivalenol, expressed in terms of kg dry malt, is shown in Figure 19.

Figure 19. Mass balance for DAS and nivalenol in brewing *(F. scirpi)* (Brew C)



The results shown in Table 26 and Figure 19 indicate that the there is a significant loss of DAS during brewing. The amount of DAS in the mash appears greater than that in the original malt. This has been observed with other brews (for example nivalenol in Brews B and D) and may be caused by sampling errors due to the heterogeneity of the mould growth in the malt. Nevertheless, there is clearly a sharp decline in concentration during mashing. There is little significant change during the later processing stages and the final beer contains less than one third of the DAS in the mash.

In this brew NIV recovery in the mash was fairly poor compared with the malt, again suggesting substantial heterogeneity of mould growth (it should be noted that the mash samples include significant proportions of solid grain material). However, it was clear that, as in Brew D, NIV survived well during brewing and the majority of that present in the malt was recovered in the beer.

4.4.2.5. Citrinin (Brew D).

The brew using *P. verrucosum*-contaminated malt was not analysed for citrinin since analyses during malting indicated that the majority of this mycotoxin was destroyed during kilning, and the concentration in the kilned malt was too low for it to be detected reliably in brewing process fractions. However, Brew D was used to assess the carry-over of FUS-X into beer, as already described in Section 4.4.2.3.

4.5 *F. langsethiae*

Towards the end of this project, the *Fusarium* species *F. langsethiae*, a known HT-2 and T-2 producer, was isolated for the first time from cereals growing in the UK. It is possible that this species may be the cause of the

sudden increase in the incidence of T-2 and HT-2 in UK cereals, first observed in the 2005 harvest. Very little is known of the behaviour of this species, therefore some additional incubation experiments were carried out to investigate conditions for growth and toxin formation. Results are shown in Table 27.

It can be seen from Table 27 that *F. langsethiae* grew well on barley at moisture levels above 20%. The appearance of the visible mould was variable, initially appearing white, but becoming green at the later stages of the incubation.

Significant quantities of both T-2 and HT-2 toxins were produced, but generally very little DON or NIV. The other trichothecenes sought (NEO, FUS-X and DAS) were not detected. Initially T-2 predominated but by the 4th week of incubation levels of HT-2 exceeded those of T-2.

More toxins were formed at 20°C than at 16°C or 25°C. Higher moisture levels generally encouraged toxin formation.

No T-2 or HT-2 toxins developed in the water control, but high levels of DON were found in these samples after 4 weeks' incubation.

Table 27. Development of toxins in barley inoculated with *F. langsethiae*

Inoculum	Moisture (approx %)	Incubation period	Incubation Temp C°	Visual growth	HT-2	T-2	DON	NIV
Water control	20	1 week	16	-	<5	<5	<5	<5
F langsethiae	20		16	-	**8**	**15**	<5	<5
	23		16	-	**5**	**8**	**31**	**6**
	25		16	-	**7**	**10**	<5	<5
Water control	20		20	-	<5	<5	<5	**15**
F langsethiae	20		20	1+ (white)	**10**	**11**	<5	<5
	23		20	1+ (white)	**18**	**27**	<5	<5
	25		20	1+ (white)	<5	<5	<5	<5
Water control	20		25	1+ (green)	**14**	**35**	<5	<5
F langsethiae	20		25	1+ (white)	**9**	**10**	<5	<5
	23		25	-	**17**	**40**	<5	<5
	25		25	-	**29**	**107**	**20**	<5
Water control	20	2 weeks	16	-	<5	<5	<5	<5
F langsethiae	20		16	1+ (white)	**19**	**19**	<5	<5
	23		16	1+ (white)	**30**	**41**	<5	<5
	25		16	1+ (white)	**59**	**60**	<5	<5
Water control	20		20	-	<5	<5	**7**	<5
F langsethiae	20		20	1+ (white)	**58**	**59**	<5	<5
	23		20	-	**90**	**129**	<5	<5
	25		20	1+ (green)	**331**	**483**	**11**	<5
Water control	20		25	-	<5	<5	<5	<5
F langsethiae	20		25	1+ (green)	**75**	**87**	**11**	<5
	23		25	-	**28**	**32**	**6**	<5
	25		25	1+ (green)	**56**	**48**	<5	<5
Water control	20	4 weeks	16	-	**5**	<5	**28**	<5
F langsethiae	20		16	-	**30**	**22**	<5	<5
	23		16	1+ (green)	**55**	**24**	<5	<5
	25		16	-	**15**	**6**	**5**	<5
Water control	20		20	1+ (green)	<5	<5	**185**	<5
F langsethiae	20		20	2+ (green)	**24**	**9**	**8**	<5
	23		20	2+ (green)	**936**	**526**	<5	<5
	25		20	3+ (green)	**1069**	**652**	**400**	**26**
Water control	20		25	-	**8**	<5	<5	<5
F langsethiae	20		25	2+ (white)	**103**	**49**	<5	<5
	23		25	1+ (green)	**75**	**22**	<5	<5
	25		25	4+ (green /sporing)	**741**	**557**	<5	**22**

5. DISCUSSION

Incidence of "emerging" mycotoxins in UK-grown cereals

The surveillance of barley, oats and wheat described in this report suggests that DAS, FUS-X and NEO are currently very rare in UK-grown cereals. Of these three mycotoxins, DAS and FUS-X were not detected in any of 84 samples from the 2004 harvest or in 83 samples from the 2005 harvest, while NEO was detected in only one oat sample out of the 167 samples cereal samples tested. Although surveillance of the 2006 harvest is not yet completed, the samples analysed to date (24 barleys, 11 wheat/wheatfeed, 10 oats/oat feed) also contained no detectable DAS, NEO or FUS-X. These results support the findings from the SCOOP report *(European Commission 2003),* which reported FUS-X in only 10% and neosolaniol and DAS in less than 5% of samples.

Incidence of T-2 and HT-2 toxins is known to be high in oats, but until very recently these toxins were hardly ever detected in UK-grown barley or wheat. However, incidence of these toxins appears to be increasing in both barley and wheat in the UK, although actual concentrations remain low. This is supported by the results so far available from the 2006 harvest, where we have detected low levels of HT-2 toxin in 15 out of 17 malting barleys (range <5 – 30µg/kg), 3 out of 7 feed barleys (range <5 – 28µg/kg), 7 out of 11 feed wheat/wheatfeed samples (range <5 – 129µg/kg) and 10 out of 10 feed oats samples (92 – 1668µg/kg).

T-2 toxin was also detected, in 8 out of 17 malting barleys from the 2006 harvest (range 5 – 13µg/kg), 2 out of 7 feed barleys (range <5 -12µg/kg), 4 out of 11 feed wheat/wheatfeed samples (range <5 – 60µg/kg), and 10 out of 10 feed oats samples (range 33 – 406µg/kg).
It is noticeable that T-2 and HT-2 tended to co-occur, but generally levels of DON in these samples were very low or undetectable.

The incidence of citrinin also seems to be relatively low (less than 10%) in commercially stored grain, significantly lower than that of OA. It is evident, however, that there is a potential for citrinin to be formed if storage is unduly prolonged. Concentrations were in the same range of those of OA. Given the recognised heterogeneity of OA contamination, and the small number of samples involved, it is not possible to be certain to what extent OA and citrinin co-occur in grain.

Growth of toxigenic moulds under laboratory conditions.

Development of toxins is known to be highly dependent on mould strain and culture conditions. The conditions used in this project (holding whole barley grain under high moisture and temperatures for up to a month) were designed to simulate the moisture/temperature conditions in the field while the crop was growing, in order to promote mould growth and toxin development. These conditions obviously bear no relationship to commercial grain storage conditions.

Under the conditions used in this project, our strains of *F. poae*, *F. culmorum* and *F graminearum* did not produce significant quantities of any trichothecenes other than DON, although they appeared to grow adequately. Consequently less common *Fusarium* species had to be used in order to obtain barley and malt with sufficiently high concentrations of toxins to allow carryover in to brewing process fractions to be followed. Both *F. sporotrichioides* and *F. langsethiae* developed significant amounts of T-2 and HT-2, and *F. sporotrichioides* (but not *F. langsethiae*) also produced neosolaniol. Neither species produced significant DON under any of the conditions tested. It was noticeable that although in most cases mould growth was favoured by higher temperatures, toxin production was often optimal in cooler conditions. This may be expected since mycotoxin formation is generally considered to be higher under stress conditions. We also observed some differences between mould behaviour on the small scale and on the pilot scale. For example, the *F. crookwellense* incubation, which developed mainly fusarenon-X and nivalenol, also contained some DAS in the small scale incubations but this was not found on the pilot scale. This emphasises the importance of environmental conditions for influencing mycotoxin formation.

Growth of the natural *Fusarium* inoculum (which tended to produce predominantly DON and NIV) appeared to be generally inhibited by the artificially applied *Fusaria* species, which would have been present at much higher concentrations. Interestingly, *P. verrucosum*, even at the high concentrations applied, appeared to allow growth of natural *Fusaria.* Ramakrishna et al *(1996)* also observed that production of T-2 toxin in inoculated grain was greater in the presence of *P. verrucosum*, although growth of *F. sporotrichioides* was significantly reduced.

Behaviour of mycotoxins during malting

The extent to which the concentration of a particular mycotoxin changes during the malting process will be affected both by the chemistry of the toxin itself (for example, solubility in water, heat stability during kilning) and by the ability of the particular mould applied to grow under malting conditions. Thus in these experimental studies, changes in toxin concentrations during the malting process may vary according to the mould species applied. The patterns observed in the experimental studies may also differ from those occurring during commercial malting, if the natural inoculum contains different mould species from those investigated here. It should also be noted that the viability and vitality of the mould inoculum would be much higher under the experimental conditions utilised in the present investigation than in commercial grain, since Fusarium moulds tend to decline during storage *(Christensen, 1963; Wallace and Sinha, 1975)*. However, any changes in toxin concentration due to the chemistry of the toxin are likely to be constant, regardless of the mould species. Concentration changes observed during steeping and kilning are most likely to be affected by toxin chemistry, while changes during germination are more likely to reflect the mould species. However, some moulds may also be capable of growth, and toxin formation, during the initial stages of kilning *(Baxter 2001)*, so concentration changes during kilning may reflect a balance between formation and thermal degradation.

The trichothecenes are generally considered to be relatively water soluble, thus some losses during steeping might be expected. However, we observed significant differences between the toxins in the extent of losses. There were significant losses of T-2 toxin, NEO, FUS-X and DAS during steeping. The surveillance results suggest that under commercial conditions HT-2 toxin is also likely to be lost, since it was apparent that both the incidence and the concentrations of T-2 and HT-2 were significantly lower in malts than in the barleys from which they were made. Losses of T-2 toxin into process water have also been observed during the wet-milling of maize (*Patey & Gilbert, 1989).* In contrast DON, NIV and the acetylated DON species were not lost to the same extent during steeping. This contrasts with studies on spaghetti cooking, where around 55% of DON present in the raw spaghetti was lost into the cooking water *(Visconti et al. 2004),* but this may be due to the difference in water temperature (steeping is normally carried out at 12 - 16°C).

DON was developed in each of the Fusaria incubations as well as from natural Fusaria present in the *P. verrucosum* incubation, where the exact species of the causative mould was not known. With *F. sporotrichioides* and the natural Fusaria there was an increase in DON concentrations overall during malting, but a reduction with *F. scirpi*, and little change overall with *F. crookwellense*.

Where NIV was present, concentrations during malting displayed a greater variation than did most of the other mycotoxins. This may represent a greater degree of heterogeneity of growth, since it is likely that the samples taken during germination were not completely homogenous (see Methods). Overall, however, there was little difference between starting barley and finished malt for the *F. crookwellense* and *F. scirpi* incubations, but a significant increase with the natural *Fusaria* inoculum.

Both HT-2 toxin and NEO displayed some capability to increase during kilning. This probably occurs during the early stages of kilning, when the grain temperature remains below about 45°C and significant moisture is still present, and has been observed previously with ochratoxin A in malt batches artificially inoculated with *P. verrucosum* (*Baxter, 2001*). Apart from citrinin, there was little evidence of significant destruction of any of the mycotoxins during kilning.

Commercially, the overall losses/increases during processing are more important than the changes occurring at individual stages. Table 28 shows the % carryover from inoculated barley at the end of the incubation period to the final derooted kilned malt.

Table 28. Carryover of mycotoxins from artificially infected barleys into kilned malt

Mycotoxin	% carryover from inoculated barley to final kilned malt					
	F sporo.	*F crook.*	*F scirpi*	*P verr.*	*Natural inoculum*	*Net formation /destruction*
DON	495	92			178	Formation
3-Ac DON		1040			263	Formation
15-Ac DON		76			65	Destruction
NIV		100	63		187	Little change
NEO	42					Destruction
FUS-X		32			26	Destruction
DAS			5			Largely destroyed
T-2 toxin	41					Destruction
HT-2 toxin	291					Formation
T-2 + HT-2	67					Destruction
Citrinin				4		Largely destroyed

This table suggests that although there is clear potential for formation of some trichothecenes during malting, others are clearly substantially reduced during processing. There is clearly potential for formation of DON and its acetylated metabolite 3-AcDON during malting. In the current studies 15-AcDON was not formed, and if initially present, generally declined during malting.

Behaviour of NIV varied with the mould species involved, but generally showed either little change overall or an increase. In contrast, FUS-X always exhibited a significant decline during malting, regardless of the mould species.

While NEO and T-2 concentrations fell significantly during malting, there was some evidence of inter-conversion of T-2 to HT-2, since the latter increased during steeping, unlike all other trichothecenes investigated. Inter-conversion of these two mycotoxins by microbial populations has been previously reported *(Kiessling et al.,1984; Swanson et al.,1987)* and for this reason it is probable that legal limits for these mycotoxins in the EU will be expressed as the sum of T-2 + HT-2 toxins. It is noteworthy that the sum of T-2 + HT-2 fell significantly during malting.

DAS and citrinin were almost completely lost during malting, with only 5% or less persisting into the finished malt.

Malt rootlets did not generally contain appreciable quantities of mycotoxins, even with these artificially infected barleys. The exceptions were nivalenol, where the concentration of toxin in the rootlets was similar to that in the derooted malt, and NEO, where the concentration in the rootlets exceeded that in the grain.

Behaviour of mycotoxins during brewing

There are several stages in the brewing process when mycotoxins could be destroyed or lost by chemical, physical or biological processes. These can be summarised as:

- Mashing – chemical hydrolysis or the action of malt enzymes
- Lautering – partitioning with the solid fraction (spent grains)
- Boiling – thermal destruction
- Whirlpool – partitioning with solid fraction (trub)
- Fermentation – action of yeast enzymes
- Racking – partitioning with the solid fraction (spent yeast)
- Filtration – physical removal by filters
- Pasteurisation – thermal destruction

The extent to which any one mycotoxin will be lost at any stage depends upon chemical and physical characteristics, mainly solubility, thermal stability, lipophilicity and chemical structure. Since the trichothecenes have a common structure there is also some potential for conversion of one moiety to a related one. It has been shown that bacteria can readily convert T-2 to HT-2 *(Beeton and Bull, 1989).*

The present investigations suggest that, although there is some potential for carryover in brewing for all the trichothecenes investigated, there are significant differences between different mycotoxins in the extent to which they can survive processing. The type A trichothecenes T-2 toxin, HT-2 toxin and DAS all showed a significant decline during mashing, although neosolaniol, which is also a Type A trichothecene, was stable during mashing.

There were also some differences between the mycotoxins in the extent to which they partitioned into the solid fractions (spent grains, trub and spent yeast). Significant losses with the spent grains were observed only for neosolaniol and T-2 toxin.

In a few cases an apparent increase in absolute toxin content was observed during mashing. These may be due to sampling errors, since it is very difficult to take a truly representative sample of mash. Since the mash sample contains both a liquid and a grain fraction, the measured mycotoxin content could be biased by a “hot spot” of mould. Niessen and Donhauser *(1993)* also observed an increase in deoxynivalenol during brewing on a pilot

scale, and suggested that it might be due to release of toxin from protein conjugates due to proteolytic activity in the mash. *De novo* synthesis by the mould is extremely unlikely under the conditions of mashing.

None of the mycotoxins was lost to any extent during boiling, supporting the general perception that these substances are heat stable. Neither were there any significant or consistent losses of any of the toxins during subsequent processing (fermentation, filtration or pasteurisation). Table 29 shows the extent of carryover of the trichothecenes investigated in this project from artificially infected malt into beer.

Table 29. Carryover of mycotoxins from artificially infected malt into beer

Toxin	% carryover from malt							
	Spent grains				Beer			
	Brew A	Brew B	Brew C	Brew D	Brew A	Brew B	Brew C	Brew D
DON	3			5	64			97
3-AcDON				0				90
15-AcDON				0				93
NIV		0	0	6		197	74	159
NEO	13				68			
FUS-X		0		0		92		114
DAS			0				47	
T-2	12				32			
HT-2	5				34			
T-2 + HT-2	9				32			

DON, NEO and the acetylated DON species all showed a high potential for carryover, ranging from 64 – 97%. FUS-X displayed an even higher carryover potential, from 92-114%. NIV was much more variable in its behaviour than the other toxins but also displayed a very high potential for carryover. In contrast, DAS, T-2 toxin and HT-2 toxin all showed significant losses overall during the brewing process.

Significant partitioning into spent grains was only observed when the malt was heavily contaminated with NEO or T-2 toxin.

6. CONCLUSIONS

The investigations described in this report suggest that the Fusarium mycotoxins NEO, FUS-X and DAS are currently very rare in UK grown grain.

The incidence of T-2 and HT-2 toxins appears to be increasing in all cereals, but concentrations remain low, except in oats.

Citrinin was not detected very frequently. However, there would appear to be a significant risk of its formation in grain which is not stored correctly.

If viable mould capable of producing NIV or DON is present on barley used for malting, it is possible that mycotoxin concentrations could rise during malting. Only limited loss of these mycotoxins during malting was observed in these experimental malts. However, most monitoring of commercial samples suggests that both incidence and concentrations of DON and NIV tend to be lower in the malts than in the starting barleys, suggesting that losses could be higher under commercial conditions where the amount of viable mould present and its vigour would be expected to be a lot lower.

In contrast, significant loss of FUS-X, NEO, DAS, T-2 toxin and citrinin are likely to occur during malting, even in the presence of large amounts of viable toxigenic mould. Under commercial conditions, with less mould inoculum, it is probable that significant quantities of HT-2 would also be lost during malting.

If present on grain used for brewing, substantial proportions of DON, NIV, FUS-X and NEO would be expected to persist into the final beer. However, significant losses of T-2, HT-2 and DAS are possible.

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Appendix 1, Sample sets collected; Malting barleys and malts

Harvest year	Date collected	Number of samples	Number of companies	Total tonnage represented	Varieties included	Growing areas covered
2004	April 2005	20 barleys 20 malts	10	No data	Decanter, Fanfare, Maris Otter, Optic, Pearl	England: East Anglia, South, Yorks. Scotland: Aberdeen, Borders, Fife, Lothians, Moray
2005	Oct 2005	18 barleys	9	50 K tonnes	Cocktail, Decanter, Optic, Pearl, Troon	England: East Anglia, Oxford, Norfolk, Shrop., Yorks. Scotland: Borders, Lothians, North East.

Appendix 2. Sample sets collected : Animal feed

Harvest year	Date collected	Cereal type	Number of samples	No of companies	Growing areas covered
2004 (freshly harvested)	Sept 2004	Barley	26	4	Scotland (5), NE (4), Midlands (5), East (4), SW (5)
		Oats Oatfeed	1 4	1	Midlands (4), East (1), SW (1)
2005 (Freshly harvested)	Sept 2005	Barley	21	3	Wales(1), East (3), NE(2), NW(3), Midlands(3), SW(3)
		Oats Oatfeed	3 5	3	Scotland(2), East (1), NW92), Midlands(1), SW(1)

Appendix 3. Sample sets collected : Milling wheat

Harvest year	Date collected	Number of samples	Number of companies	Varieties included	Location
2004	Nov 2004	25	5	Claire, Consort, Cordiale, Einstein, Hereward, Malacca, Napier, Robigus, Soisson, Solstice, X-9, Xi-19,	North West, Midlands, East, South East, South west.