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# Mycotoxins that affect the North American agri-food sector: state of the art and directions for the future

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**REVIEW ARTICLE**

## Abstract

This paper summarises workshop discussions at the 5th international MYCORED meeting in Ottawa, Canada (June 2012) with over 200 participants representing academics, government and industry scientists, government officials and farming organisations (present in roughly equal proportions) from 27 countries. Workshops centred on how mycotoxins in food and feed affect value chains and trade in the region covered by the North American Free Trade Agreement. Crops are contaminated by one or more of five important mycotoxins in parts of Canada and the United States every year, and when contaminated food and feed are consumed in amounts above tolerable limits, human and animal health are at risk. Economic loss from such contamination includes reduced crop yield, grain quality, animal productivity and loss of domestic and export markets. A systematic effort by grain producers, primary, transfer, and terminal elevators, millers and food and feed processers is required to manage these contaminants along the value chain. Workshops discussed lessons learned from investments in plant genetics, fungal genomics, toxicology, analytical and sampling science, management strategies along the food and feed value chains and methods to ameliorate the effects of toxins in grain on animal production and on reducing the impact of mycotoxins on population health in developing countries. These discussions were used to develop a set of priorities and recommendations.

**Keywords:** mycotoxins, USA, Canada, toxicology, mycology, crop production

## 1. Introduction

This paper provides a 'state of the science' discussion on how mycotoxins in food and feed affect value chains and trade in the region covered by the North American Free Trade Agreement (NAFTA). This meeting was held in Ottawa, Canada in the North American regional meeting of MYCORED (www.mycored.eu). Key discussion points were

summarised by workshop chairs, and then used to underpin the development of a set of priorities and recommendations, targeted at government, industry and academia in NAFTA as steps forward in a research agenda.

## 2. Mycotoxin sampling and analysis

The detection of one or more mycotoxins above specifications or legal tolerances in a commodity or a food product often triggers a complex series of actions that can prove costly for industry, regulators, and ultimately, consumers. There is a benefit to industry and the public to prevent a non-compliant commodity from entering food and feed channels. However, the lower the tolerance, the more challenging it becomes to minimise the likelihood of false positive or false negative detection results. Ochratoxin A (OTA) was identified as particularly challenging to manage in cereal-based food products (particularly those destined for infant foods) because these products must meet tolerances in the low µg/kg range. OTA was confirmed to be heterogeneously distributed in unprocessed cereals, and generally at very low concentrations when present.

Sampling methods and measurement uncertainty due to sample heterogeneity remain two of the greatest challenges when managing mycotoxins at concentrations in the low µg/kg range. Sampling bulk grains for mycotoxins requires a well-characterised sampling plan with an understanding of their distribution in the larger bulk. For aflatoxins and deoxynivalenol (DON) in maize, and DON in wheat, reliable information exists (Whitaker, 2006). In the case of OTA, Dr. Thomas Whitaker provided preliminary information on an operating curve for this mycotoxin in wheat and oats from a large Canadian study. Based on the study's particular sampling and sample preparation, a representative 2 kg laboratory sample was needed for truckload deliveries of raw cereal grains, followed by analysis of a 100 g test portion in order to minimise false positives and negatives.

Analytical requirements vary at the many points of sale along food production chains, including the harvested crop, grain trading points, processed ingredients (e.g. flour) and finished food products. Primary processors need rapid compliance testing of raw ingredients for diversion to appropriate uses, whereas regulators assessing population exposure require mycotoxin analyses. The analyses required in these two cases will have some similar needs, such as proper sampling, to obtain representative portions for analysis, as well as accurate and precise results. However, the appropriate technology that meets the various needs would differ. A disposable lateral flow device could meet the needs of the primary producer or processor, whereas comprehensive instrumentation such as a liquid chromatograph tandem mass spectrometer (LC-MS/MS) would be most appropriate for the requirements of the regulator. Three main topics were covered in the workshop discussions: sample preparation, simple screening technologies (including planar waveguide, Fourier transform-near infrared, aptamers, ELISA, fluorescence polarisation, and multiplex lateral flow assays), and comprehensive diagnostic technologies (including immunoaffinity column preparation with HPLC, HPLC-fluorescence detection, and LC-MS/MS).

Proper sampling is the basis of good analysis. Many studies highlight the fact that primary sampling from the bulk is the largest source of variance and uncertainty in results, due to the heterogeneity and low concentrations of analyte in bulk samples (Whitaker *et al.,* 2010). The largest decrease in uncertainty can be achieved by modifying primary sampling; adjusting sample preparation or the analytical test method leads to a more modest reduction in uncertainty of test results.

The complexity of food production chains – in addition to the challenges inherent in sampling heterogeneously distributed low concentrations of mycotoxins in bulk samples – means that there is no 'one size fits all' sampling plan for all mycotoxin/commodity combinations. Participants in the discussion also explored the possibility of directing resources required to effectively sample and analyse raw commodities into the prevention of mycotoxin formation, which would push mycotoxin testing to earlier stages of the value chain, such as on-farm. This scenario has both advantages and disadvantages, and these vary according to the stakeholder and their point of view. However, there was common agreement that premiums are required to reinforce appropriate practices (whether they are to prevent mycotoxin formation or to conduct proper sampling and analysis) along the food production chain.

The importance of sampling and how sample heterogeneity affects the variance and uncertainty of testing results is still largely misunderstood. Specific comments such as 'the LC method was not reliable for OTA analysis' or 'testing methods are not accurate enough' blur the issues of accuracy and precision due to analytical method versus the effect of sample heterogeneity on these method performance characteristics. The existence of these misconceptions emphasises the urgent need to keep discussing the importance of accurate sampling as well as the need to develop and provide clear guidance for organisations without their own sampling expertise. A positive step was news that a publicly available on-line tool is being developed by Drs. Thomas Whitaker and Andrew Slate (North Carolina State University, USA) to calculate the performance of mycotoxin sampling plans for various mycotoxin/commodity combinations. The users will be able to input sampling plan design elements such as sample size and accept/reject limits and the model will calculate an operating characteristic curve that will give an indication

of the probability that good lots will be rejected and bad lots accepted by the sampling plan design.

Many tests are labelled as 'rapid', yet different points along the food production chain have different time constraints for obtaining useful test results, creating different meanings for the term 'rapid' for different stakeholders. It was also clear that advertised test times often failed to take into account the time for sample collection, processing and extraction. Some industry participants felt that in certain situations 30 min was not a fast enough turnaround time for test results for their current processes, and those representing grain elevators highlighted the need for results in less than 10 min to manage truck deliveries.

A number of different analytical techniques were discussed for routine analysis ranging from established methods such as ELISA (Meulenberg, 2012; Zheng *et al.,* 2005) to potentially newer techniques involving optical devices (Caputo *et al.,* 2010), methods based on novel spectroscopic techniques (Piletska *et al.,* 2010) as well as those based on aptamers (McKeague *et al.,* 2011). As they emerge, there is an urgent need to have the performance of novel methods characterised with regards to linearity, selectivity, recovery, repeatability, reproducibility, sensitivity, and matrix effects, and then validated in interlaboratory studies, as well as evaluated for use in non-laboratory settings. As of September 2013, only 8 rapid test kits for mycotoxins had gone through the AOAC International performance-tested method process. The European CON*ff*IDENCE (www.conffidence.eu) program has developed and evaluated a multi-mycotoxin dipstick assay for zearalenone (ZEA), T-2 and HT-2 toxins, DON and fumonisins (Lattanzio *et al.,* 2012). The US Department of Agriculture agency, GIPSA, has the Performance Verified system. Of the rapid tests for mycotoxins, 19 quantitative and 23 qualitative tests for various mycotoxins are approved by GIPSA.

Various researchers in the European Union have contributed to the development of programmes to encourage/require validation. The Monitoring and Quality Assurance in the Total Food Supply Chain initiative (MoniQA; www.moniqa.org) is active in promoting these goals. Overall though, the use of analytical methods is effective only to the extent that proper sampling and sample preparation is performed to obtain a representative sample of the bulk commodity. Analyses for mycotoxins early in the value chain are also most useful when used with good farming practices or Hazard Analysis Critical Point (HACCP) systems (Aldred *et al.,* 2004; Senyuva and Gilbert, 2011). Some commercially available test kits or other methods using simple technology, including the majority of novel test methods discussed during the workshop, may require the use of solvents such as methanol to extract mycotoxins from samples. Regulations often mandate the use of laboratory fume hoods to protect the health and safety of employees using such solvents. This type of infrastructure is not feasible at many primary production facilities. Some kits have recently been validated with 50% ethanol:water which may alleviate the difficulties of working with solvents in field applications.

There are also sample preparation techniques that are feasible in a laboratory setting, but not at points early along the food production chain. One specific example raised during the workshop pertained to using slurry mixing to prepare a sample for mycotoxin analysis. Grain elevators strive to keep their facilities dry to prevent mould growth; therefore, the use of water in preparing samples for analysis is not ideal.

These feasibility challenges in non-laboratory environments indicate a need to explore opportunities to 'piggyback' on other analyses that could be happening at the same point in the food production chain. For example, rapid visual analysis is often performed at primary and subsequent production facilities for grain quality assessment. The question was raised as to whether or not the same sample could then be used for mycotoxin analysis. If these novel routes for sample preparation appear to be feasible in non-laboratory environments, they will require evaluation and validation to ensure that accurate and precise mycotoxin test results are obtained.

Monitoring for contaminants in food, including mycotoxins, is required when commodities and food products have to comply with regulations. However, due to the sampling and analytical challenges discussed in the workshop, there are legitimate questions that need to be answered as to the most effective point along the value chain to perform monitoring. Developments in sampling and analytical methods with improved performance and reduced costs are slowly improving options for monitoring mycotoxins, but more investment in education during crop production and storage is needed to prevent contamination as there are no analytical methods that would allow mycotoxins to be managed by detection.

## 3. Toxicology

From the earliest studies of mycotoxins, mixtures of compounds were recognised to be important in their toxicity (e.g. ergotism; Barger, 1937; aflatoxins; Newburne and Butler, 1969). For aflatoxins, much later analyses revealed that the symptoms described during the initial outbreaks were a combined result of aflatoxins and cyclopiazonic acid (Cole, 1986), which was confirmed in an authentic sample of the original material (Bradburn *et al.,* 1994). Despite this understanding, there has been little effort to evaluate mixtures in a critical fashion. In a review by Grenier and Oswald (2011), many of the interactions in the original publications cited in the review are not supported by appropriate statistical tests. The majority of

combinations tested are not realistic. The public health implications of mixtures have mainly been speculative. In Ottawa, a set of criteria was proposed to provide some focus for this potentially important discussion. The focus of mixture studies should be for those mycotoxins that commonly occur in food or feed, such as fumonisin and aflatoxin or fumonisin and DON in maize. In these cases, the mode of action and key events are individually known. Biomarkers of exposure need to be used to measure internal dose and mechanism-based biomarkers should be utilised to differentiate between dose-additive and response-additive interactions. In animal studies, these must be designed to provide dose-response information for both pathology and biomarkers.

Renewed interest in revising the regulations for the toxins DON and OTA resulted in a particular focus on these toxins. The Joint FAO/WHO Expert Committee on Food Addititives (JECFA) provisional maximum tolerable daily intake (PMTDI) for DON is based on the anorexic and growth effects in a two-year mouse study (Iverson *et al.,* 1995). Originally, Prelusky (1997) suggested that the effects were mediated by altered neuroendocrine signalling within the nervous system, but since then DON has been shown to affect the circulating insulin-like growth factor (Amuzie *et al.,* 2011) and induce cytokine up-regulation in parts of the murine brain, which is related to changes in feeding behaviour and body temperature (Bonnet *et al.,* 2012). DON is also known to cause emesis in swine, mink and dogs (Wu *et al.,* 2012), an effect also observed in humans (Miller, 2008). Anorexia is caused by induction of gut satiety hormones (Flannery *et al.*, 2012), whereas emesis is caused by release of these hormones in addition to the neurotransmitter serotonin (Wu *et al.*, 2013).

For economically important mycotoxins, data supporting hazard identification and characterisation have been generated by multiple laboratories over a relatively long time period with little or no coordination. Meanwhile, actions taken by regulatory authorities to manage risks associated with mycotoxin exposure make use of the best available data at the time of decision-making. Thus, new insights into mycotoxin toxicity and mode of action (MOA) emerging from laboratories all over the world play a key role in mycotoxin risk assessment. In the workshop on mycotoxin hazard, assessments were considered from the perspectives of (1) new tools for mycotoxin hazard identification; (2) new data on mycotoxin metabolism and toxicity; and (3) advances in biomarkers for mycotoxin exposure.

Despite the active regulatory process for OTA, the mode of action for OTA is still unresolved. The best evidence is that once inside the cell, OTA may have multiple MOAs and targets (Mally, 2012). It appears that some, if not all, biochemical lesions resulting from OTA exposure have a dose threshold. There are many questions about the existing whole animal data and most of the recent studies are not adequate for regulatory use. There was little consensus on the quality of the information available at this time to make a major regulatory decision given the scale of the North American grain production systems.

OTA risk assessment was a key topic of interest stemming from a publication by Kuiper-Goodman *et al.* (2010), in which the tolerable daily intake for OTA was re-evaluated based on consideration of the MOA for OTA (as it was understood in 2010) and on a probabilistic exposure assessment. Data were presented describing kidney gene and protein expression, respectively, in cancer-prone p53 heterozygous mice exposed to OTA in diet for 26 weeks. This model has been used to generate chronic toxicity data for several mycotoxins, such as described by Bondy *et al.* (2012) for fumonisin $B_1$ ($FB_1$). Evaluation of the p53 heterozygous mouse for use in carcinogenicity testing indicates that this model consistently develops tumours in response to genotoxic but not non-genotoxic carcinogens within a 26-week exposure period (French *et al.,* 2001). Toxicologic, pathologic and genomic analyses of tissues from chronically exposed p53 heterozygous mice added to the scientific evidence supporting OTA nephrotoxicity, but failed to clarify the MOA for OTA carcinogenicity. Protein biomarkers of renal injury were significantly expressed in mice exposed to OTA, consistent with nephrotoxicity at the level of the proximal tubule. Changes in gene expression in kidneys from OTA-exposed mice were most pronounced in canonical pathways related to oxidative stress, cellular growth and proliferation. Rapidly changing technologies for assessing global changes in gene expression were discussed. This included the trend to move away from microarray approaches and towards next-generation DNA sequencing. Nevertheless, handling and storing vast amounts of data and extracting biologically relevant insights from data analyses remains a challenge for toxicologists.

Many *in vitro* genotoxicity/mutagenicity assays have been conducted to determine whether or not OTA is genotoxic. Adding to the general confusion on the mode of action of OTA, bacteria-based assays such as the Ames test are negative and assays based on mammalian cells are weakly positive, depending on variable assay conditions across laboratories. This was emphasised in the presentation by T. Schrader (Health Canada), who found OTA to be weakly mutagenic in a hamster V79-HGPRT assay when cells were transfected with a CYP1A2 expression vector, and non-mutagenic in the Ames assay using multiple *Salmonella* strains. Assays using human mammary adenocarcinoma cells transfected with human CYPs indicated that OTA mutagenicity in mammalian cells may require metabolism; however, this finding is not reproducible in bacterial cells supplemented with microsomal S9 preparations. Although data from genotoxicity assays are equivocal for OTA, *in vitro*

approaches continue to be useful for identifying mycotoxin metabolites and for characterising specific cellular events associated with mycotoxins and their metabolites.

The dual nature of DON immunotoxicity (stimulation and suppression) is the rationale for studies on the cytopathic effects and replication of Porcine Reproductive Respiratory Syndrome Virus (PRRSV) *in vitro*. DON dose-dependently affected the survival of PRRSV-infected cells and replication of the virus. The toxin further increased the expression of interferon-α but decreased the expression of interferon-β and tumour necrosis factor-α. It was noted that there are many challenges when conducting immunotoxicity studies in lab animals and these are accentuated when moving to larger outbred species in a farm setting. Nevertheless, it was agreed that such studies in swine can provide important information for risk assessment.

Hepatic microsomal preparations from several species were used to identify DON-glucuronide conjugates and to explore species differences in DON metabolism. The workshop considered the feasibility of this approach for identifying other metabolites, such as sulphate conjugates, as well as the confounding factors associated with detecting DON conjugates in plasma and other biological matrices. The latter is important as a step in developing biomarkers for mycotoxin exposure, a necessity for epidemiology studies.

Ideally, mycotoxin biomarkers should be readily detectable in an accessible sample such as human urine. Dietary exposure to DON has been correlated with free DON plus DON-glucuronide conjugate in urine samples (Turner *et al.,* 2012). Development of a plasma biomarker for DON is complicated by protein binding (Meky *et al.*, 2003). At the meeting, Dr. Turner noted that protein-bound DON may be a useful biomarker for long-term exposure.

Urinary biomarkers for multiple mycotoxins, including DON, have been used successfully in human epidemiology studies. Fumonisin, DON, nivalenol, OTA, ZEA and its zearalenol metabolites were all detected in urine samples from rural South African subsistence farmers. Aflatoxins were not detected in urine due to their absence in home-grown maize from the study area. A number of factors were discussed in the context of variability in urinary mycotoxin biomarker levels, including differences in the source and quantity of home-grown maize consumed, inter-individual differences in toxin metabolism and excretion, and differences related to the temporal distance between exposure and sample collection.

Based on the range of mycotoxin-related issues discussed, several general conclusions and/or future actions were evident. For OTA, the current status is that MOA data suggest both genotoxic and non-genotoxic MOAs may contribute to the observed toxic effects, which has led to contention over the most appropriate risk management decisions. Further data generation on the MOA for OTA using animal and cell-based assays would be useful. Furthermore, given the complexity of the immunotoxic effects of trichothecenes and other mycotoxins, *in vivo* studies are necessary to validate mechanisms discovered *in vitro* and to inform risk management. Biomarker development for all mycotoxins is a valuable tool for epidemiological studies, linking exposure to effect. Detection of mycotoxins and their metabolites in urine is being used effectively, whereas detection in blood and other biological matrices can be challenging. Development and validation of functional biomarkers specific for mycotoxin exposure is a growth area.

## 4. Mycology and fungal genomics

There has been considerable investment in genomics of the toxigenic fungi and the use of sequencing as an alternative to morphological species identification. Primarily because of the economic importance of the toxigenic *Fusarium, Penicillium* and *Aspergillus* species, reproducible species concepts are critical to commerce, managing genetic resistance, and studies to manage climate change. While a number of major academic and government laboratories in most countries have access to cost-effective DNA extraction and gene sequencing facilities, these are not widespread. Workable and reliable methods for species identification, e.g. the use of multiple growth media/ skilled microscopy (see, for example, Varga *et al.,* 2011) remain indispensable for people who need to study the prevalence and agricultural ecology of these fungi. For some applications such as monitoring and tracking, the use of genomic probes during sampling and for research studies of agronomic or storage factors is showing promise as an alternative to culture-based studies. This requires improvements in sampling, extraction and the application of reliable probes or sequences. Proof of concept has been obtained using older qPCR-based methods (e.g. Demeke *et al.,* 2010). However, the opportunities afforded by massively parallel sequencing (e.g. Das *et al.,* 2008) will produce methods with practical utility as the cost of such sequencing decreases.

For the pathogenic *Fusarium graminearum*, a sophisticated understanding of gene and proteome regulation during pathogenesis, production of conidia or ascospores and toxins helps identify genes with specific functions (Hallen-Adams *et al.,* 2011; Sikhakolli *et al.,* 2012). This has been effectively studied in barley, maize and wheat. Along with understanding transcriptional shifts to identify genes of interest, more reliable identification can be made of important versus minor quantitative trait loci (QTL) (Rutkoski *et al.* 2012).

Genetic studies of *Aspergillus flavus* and *Aspergillus parasiticus* over the past 25 years have provided valuable knowledge on aflatoxigenicity, ecology, and host colonisation. Global studies of *A. flavus* and *A. parasiticus* revealed that in cooler wet areas, atoxigenic strains predominate in clonal *A. flavus* populations and toxigenic strains predominate in warm dry areas (Carbone *et al.,* 2010; Moore *et al.,* 2013). In fertile intra- and interspecific crosses, there is both qualitative and quantitative variation in aflatoxin chemotypes ($B_1$, $B_2$, $G_1$, $G_2$), *O*-methylsterigmatocystin and cyclopiazonic acid (Fisher and Henk, 2012; Olarte *et al.,* 2012; Worthington, 2011).

In addition to ground-breaking ecological studies, detailed information on the genomics of *A. flavus* has permitted some innovative studies on colonisation. Resistance to *A. flavus* has been the subject of speculation for 30 years (e.g. Payne, 1992). It had been thought that *A. flavus* was a saprophyte growing on stressed plants. However, more recent studies indicate that the fungus shows tissue-specific colonisation in maize. In maize, typical defence genes are expressed in advance of *A. flavus* hyphae and the ethylene-inducing peptide (nepA) modulates susceptibility. Drought stress impairs expression of known resistance genes. Transcriptional analyses of maize and *A. flavus*, during infection of maize kernels, have revealed a number of potential pathogenicity factors for *A. flavus*. The transmembrane protein pth11 showed more than a 2-fold expression difference when grown on living maize tissue while nepA expressed more than a 3-fold change (Reese *et al.,* 2011).

The overarching question of how our understanding of mycotoxin biosynthetic pathways, environmental conditions, and microbial community composition informs management strategies for reducing mycotoxin contamination arose out of the discussion. The genes for aflatoxin biosynthesis in *Aspergillus*, fumonisin and trichothecene biosynthesis in *Fusarium*, and fumonisin production in *Aspergillus niger* and other black Aspergilli are clustered (Woloshuk and Shim, 2012). This may facilitate the coordinated expression and regulation of these genes, and, if correct, raises questions about the minimum number of genes that comprise a cluster and whether a single gene module comprising two divergently transcribed genes represents the earliest stages of functional innovation and the birth of clusters. In the aflatoxin gene cluster, several genes are organised in modules such as *aflA/aflB*, which exists as a gene module in many *Aspergillus* species (Carbone *et al.,* 2007). Recruitment of gene modules may represent the earliest stages of cluster formation. In *A. flavus,* four genes are clustered in cyclopiazonic acid biosynthesis; similarly four genes are involved in butenolide biosynthesis in *Fusarium* and about 4-5 genes are involved in OTA biosynthesis in *A. niger* and *A. carbonarius*. This modular design suggests that gene clusters may have originated from recruiting several smaller gene modules. Cluster disassembly may follow a similar pattern, but in reverse, such that partial gene clusters represent intermediate snapshots of cluster evolution.

Another interesting question is why gene clusters persist in fungi when they no longer make toxin. One possibility is that partial or missing gene clusters are relics of a functional gene cluster or have been preserved as a result of selection on some important non-toxigenic trait. For example, it is not uncommon to find a mix of aflatoxin-producing and non-aflatoxigenic *A. flavus* strains in a single field population. This natural equilibrium may explain why biocontrol methods using non-aflatoxigenic *A. flavus* strains can work. However, non-aflatoxigenic strains can be genetically very different and it is important to distinguish between (1) non-producing strains that are missing genes or the entire gene cluster; (2) non-producers that harbour a single nonsense mutation; and (3) non-producers with multiple loss-of-function mutations in random cluster genes (e.g. Chang *et al.,* 2012). There is mounting evidence that these three mutation classes show highly variable geographic distributions and may function as *A. flavus* ecotypes. Similar phenomena are observed in fumonisin-producing *Aspergillus* species. In *A. niger* and other black Aspergilli there is an approximately even split in occurrence of fumonisin producers and non-producers.

A study of fumonisin gene clusters in non-producers showed multiple genotypes that may harbour loss-of-function mutations; moreover, many non-producers are missing key genes within the fumonisin gene cluster. Gene loss appears to be species-specific such that patterns of gene deletions in the fumonisin cluster can be useful in separating *A. niger* from *Aspergillus awamori.* A perhaps related phenomenon has been observed in the trichothecene gene cluster of *Fusarium* species, such that species can be separated based on two forms of the trichothecene biosynthetic intermediate 3,15-deacetyldeoxynivalenol deacetylase gene (*TR18*), which is responsible for conversion of 3,15-diactetyldeoxynivalenol to acetyldeoxynivalenol trichothecene chemotypes. The implication is that toxin chemotypes may play an important role in lineage and species diversification. An interesting hypothesis is that chemotype plasticity may be important for fungal adaptation to changing environmental conditions. The prediction is that one would expect to find the greatest diversity in chemotypes in the most heterogeneous environments.

Environmental conditions, such as temperature, light and water activity influence the severity of mycotoxin contamination. For example, *in vitro*, growth and fumonisin production in *Fusarium verticillioides* was shown to be stimulated in the presence of visible light of all wavelengths compared to dark incubation. Fumonisin production

decreased significantly when the intensity of blue light was increased, as well as under short wave blue light (e.g. Fanelli *et al.,* 2012). Climatic zones may further impact the biology of mycotoxigenic fungi, and in particular, their capacity to reproduce clonally or sexually which can potentiate or alleviate mycotoxin contamination. A study of mating types of *A. flavus* in warm temperate North Carolina (USA) showed that the predominant mating partner is putatively heterokaryotic for mating type genes *MAT1-1* and *MAT1-2*. In contrast, *A. flavus* populations in subtropical climatic regions such as Georgia, India, Australia and Benin are predominantly homokaryotic for mating type. Interestingly, in *A. flavus* we see a latitude gradient where the more temperate populations are non-toxigenic. Perhaps these fungi are functionally homothallic in more temperate regions where overwintering significantly reduces population numbers and self-fertility is the best way to assure fungal growth and dissemination. Or perhaps there are other biotic and abiotic interactions that influence mating potential in fungal populations. Species diversity and biotic interactions, such as insect predation of plants, are known to be higher in the tropics compared to temperate regions, so one cannot rule out that the latitudinal toxin gradient may be more of a latitudinal diversity gradient.

Host-plant interactions may also play an important role in fungal life cycle control and mycotoxicity. For example, oxylipins have been implicated in altering the ratio of asexual to sexual spore development and oxylipin production in host plants has been linked to triggering fumonisin biosynthesis via histone acetylation and other epigenetic processes (e.g. Scala *et al.,* 2013). One potential biocontrol application is to develop methods that target histone deacetylation to prevent fumonisin production, and a future research focus is to examine how oxylipin affects fumonisin biosynthesis at an epigenetic level. Many more factors and interactions may be involved in the micro-ecosystem and it is clear that we need to move beyond each individual factor and examine ecosystem-level functions, such as water and soil chemistry, and microbial community composition to fully understand mycotoxicity.

## 5. Distribution of mycotoxigenic fungi and host plant resistance

Both *Gibberella* ear rot (GER) (*F. graminearum*) and *Fusarium* kernel rot (*F. verticillioides*) have complicated natural histories. Various insects and environments (drought, temperature) condition the plant disease response to the fungus. Host resistance to insects or to environmental stresses confers *de facto* resistance to the disease either by quantitative genetic traits or broader hybrid characteristics. This includes such things as pericarp thickness, kernel morphology, maturity class, flowering duration, early grain filling, and dry down rate (Parsons and Munkvold, 2012). Transgenic hybrids with Bt events that manage ear-feeding *Lepidopteran* insects have the effect of lowering fumonisin and DON when the insect causing ear damage is controlled by the transgene (Folcher *et al.,* 2010; Hammond *et al.,* 2004). Private maize breeders have a deep knowledge of the characteristics of their germplasm stretching back in some cases for nearly a century. To respond to the GER challenge (particularly relevant for the north eastern areas) tolerance has been sought from stiff stalk and non-stiff stalk landraces and exotic germplasm. This has made use of molecular markers and QTLs in breeding and cloning or introgression of resistance genes and doubled haploid technology. After a serious epidemic in 2006 in the Great Lakes region of North America, one of the seed companies conducted an extensive review of their germplasm and discovered a linkage of high susceptibility to GER in one of their hybrid families and it was recommended that this family no longer be marketed in this region. Annual yield increases in maize continue to surprise producers and there is no sign that these yield gains are slowing down. Ironically, the highest yielding hybrids tend also to be susceptible to GER, and higher levels of resistance may result in a trade-off for yield, due mainly to the complexity and number of genes involved. Even more ironic is that weather that favours a high yielding crop in the Great Lakes region also favours a GER epidemic in the region.

Achieving resistance to GER in maize hybrids is a goal with several challenges. In addition to the yield penalty associated with some resistance genes, the quick turnover (short market life) and sheer number of new hybrids reaching market annually makes a programme of testing and comparison over years and environments insurmountable. On the flip side, if the focus of attention was placed on developing a system to keep the highly susceptible hybrids out of the high risk regions, local and broader epidemics may be easier to manage. In 2006, over 60% of the market share was held by only a few hybrids, which, while very high yielding, were also the most susceptible among those grown in the region. Furthermore, recent success was reported at the meeting in the use of fungicides (metconazole and prothioconazole) in combination with less susceptible hybrids to reduce both the level of GER and DON in harvested grain, suggesting that some progress is possible to manage this disease. This is especially true for the Great Lakes region, where the problem of DON contamination has shifted from mainly being a swine producers' problem to a problem for cash crop producers. It was clear in 2006 and again in 2011 that DON is a real and present problem in the industrial maize sector, when more than 40% of 1,768 commercial maize samples from Ontario (Canada) tested by the University of Guelph contained DON concentrations above 3 mg/kg. As with previous studies (Schaafsma and Hooker, 2007), the main factors of these epidemics remain hybrid susceptibility to *F. graminearum* and weather (precipitation and temperature). Resistance in both wheat and maize is usually conditioned by multiple genes and

inheritance of resistance is complicated or associated with lower yield. Because of the sporadic nature of mycotoxin outbreaks, crop producers continue to prefer selecting high-yielding hybrids rather than resistant ones that may have lower yields in years of low disease intensity.

Tools to speed up the process of identifying and utilising sources of resistance (double-haploid technology, year-round nurseries, molecular markers, more precise, higher-throughput phenotyping methods) must be developed, optimised and implemented to the fullest extent possible. Finally, the role of biotechnology must be acknowledged and embraced. Gene combinations including conventional resistance to mycotoxigenic fungi and transgenic resistance to insects have the potential to be highly effective, particularly for fumonisins.

For the past 30 years, public breeding of maize in Canada has concentrated on a mixture of inbred development and basic research to improve resistance to GER. This has involved optimising methods for GER field screening, resulting in the release of ~10 inbreds with improved resistance. One of the early inbreds (Reid *et al.,* 2003) is a common resistant check in many programmes. Some of these inbreds have resistance to multiple diseases (Reid *et al.,* 2009). The availability of these inbreds has allowed researchers to explore QTLs, underlying mechanisms and inheritance of resistance (Mesterhazy *et al.,* 2012).

As in wheat, the molecular mechanisms for resistance to *F. graminearum* in maize have not been determined. An update was presented on research to characterise and map silk and kernel resistance in the GER-resistant maize inbred CO441 (Reid *et al.,* 2002). Proteomic profiling demonstrated that CO441 kernels contain higher levels of defence-related proteins, perhaps providing a basal defence against *Fusarium* infection (Mohammadi *et al.,* 2011). A B73 X CO441 $F_6$ recombinant inbred population of 419 lines has been phenotyped for kernel and silk channel resistance over two field seasons and association mapping of this resistance is underway using genotyping by sequencing data (L. Harris and E. Buckler, personal communication). Proteomic analysis between susceptible and resistant maize lines has identified several factors that may impart resistance directly or indirectly against fungal invasion and aflatoxin formation. Some of these factors map to QTLs associated with resistance (Brown *et al.,* 2010). Phenotype selection is the primary tool for improving *Fusarium* Head Blight (FHB) resistance from native sources of wheat. In the USA/Canada, older germplasm has so-called native genes for resistance (minor genes) with a smaller but additive effect to produce partial resistance. These accumulated in germplasm developed for the maize belt over time (Groth *et al.,* 2011). Good progress has recently been obtained using phenotype selection (in FHB nurseries) combined with molecular-assisted breeding (Mesterhazy *et al.,* 2012).

One of the mechanisms of FHB tolerance that has received considerable attention recently is the formation of DON glucoside which lacks the protein synthesis-inhibiting properties of DON (Boutigny *et al.,* 2008), leading in some cultivars to the accumulation of high concentrations of DON glucoside not detected by most analytical methods. Since the discovery of this phenomenon 25 years ago, a number of additional glucosides have been discovered including those of other trichothecenes (Berthiller *et al.,* 2013). These findings have been generated by the measurement of these metabolites in plant tissues. The original work used $^{14}$C-DON, which provided direct evidence of metabolism by the plant (Miller and Arnison, 1986). Conceptually similar experiments have recently been reported using isotope-assisted untargeted metabolomics. Wheat heads were treated with 100 µg of 1+1 (v/v) mixture of non-labelled and fully $^{13}$C-labelled DON. The analysis revealed DON and DON-3- glucoside. However, additional, new conjugates were discovered including for the first time a DON-glutathione conjugate and indications of other glutathione-S transferase DON conjugates (Kluger *et al.,* 2013). These direct chemical approaches including the use of $^{13}C$ $CO_2$ of growing wheat plants in growth chambers will provide direct evidence of the metabolic process for this and other *Fusarium* toxins.

The workshop discussion considered material on the geographic distribution of mycotoxigenic fungi and their mycotoxins as well as efforts to understand host-plant resistance. Many of the talks highlighted concerns of multi-toxin contamination and the impact of climate change and global trade on pathogen/mycotoxin profiles. An extensive collection of strains of *Aspergillus, Fusarium* and *Penicillium* species and other genera received from national and international fungal culture collections and from samples of cereals, grape and dried fruits have been collected worldwide by the MYCORED project. *Fusarium ramigenum* and strains provisionally identified as *Fusarium proliferatum* are known from dried figs contaminated by fumonisins from Italy (Moretti *et al.,* 2010). This finding was extended to other fig-producing areas of the Mediterranean (e.g. Heperkan *et al.,* 2012). Similarly, a chemotype map of *F. graminearum* and *Fusarium culmorum* throughout European wheat was generated using PCR genotyping, showing a predominance of 15- acetyldeoxynivalenol (ADON)-producing *F. graminearum* isolates and nivalenol-producing *F. culmorum* isolates (Vogelgsang *et al.,* 2009).

The aggressiveness and trichothecene production of 3-ADON versus 15-ADON *F. graminearum* genotypes remains controversial (Gilbert *et al.,* 2010; Von der Ohe *et al.,* 2010). Studies at the University of Guelph reported that, using a susceptible winter wheat cultivar, four Canadian *F. graminearum* 3-ADON-producing strains resulted in both a higher FHB rating and DON concentration relative to four 15-ADON-producing isolates.

Based on previous work that suggested antioxidants play a role in mycotoxin production and FHB susceptibility, researchers at Carleton University (Canada) compared phenolic levels between three infected and uninfected winter wheat cultivars. They found that the distribution of phenolics was significantly different between infected cultivars. Phenolics previously reported to inhibit Tri5 (Boutigny *et al.,* 2010) were present at higher concentrations in some moderately resistant cultivars. To screen for cellular targets of the suite of secondary metabolites secreted by *F. graminearum,* a gene deletion collection of *Saccharomyces cerevisiae* was used to screen for essential and non-essential genes affected by the culture filtrate of *F. graminearum*. Genes showing resistance or sensitivity to these metabolite mixtures could be candidates for developing resistance to *Fusarium* infection.

Responding to the lack of data on the occurrence of the OTA-producing fungus *Penicillium verrucosum* from Ontario storage bins, researchers at the University of Guelph reported on the incidence of the fungus and OTA in 40 commercial winter wheat storage bins located in southern Ontario. Dilution plating of the 40 samples showed 28% were positive for *P. verrucosum*. Of 92 isolates tested for mycotoxin production *in vitro*, 83 and 98% were capable of producing OTA and citrinin, respectively. Only one sample out of 40 tested positive for OTA by LC-MS/MS. However, the presence of inoculum of the fungus in OTA-negative bins could increase the risk of OTA contamination of wheat grains under conducive environmental conditions. Condensation in the granary was the key contributor to the occurrence of OTA in the wheat sampled (R.R. Burlakoti, V. Limay-Rios and A.W. Schaafsma, personal communication).

## 6. Managing mycotoxins pre- and post-harvest

Presentations related to this workshop focused on management tactics for mycotoxigenic fungi, as well as multi-trophic interactions involving these fungi, interactions that both complicate and inform management strategies. Pre-harvest mycotoxin management emphasised genetic resistance, insect management, the development and use of fungicides, cultural practices, biological control methods, and the integration of genetic and chemical approaches.

The risk of mycotoxin contamination in maize can be influenced heavily by insect activity. This is clearly an important factor in relation to fumonisin and aflatoxin contamination, but insects also can influence contamination by DON and other mycotoxins (Abbas *et al.,* 2007; Folcher *et al.,* 2010, 2012; Hammond *et al.,* 2004; Schaafsma *et al.,* 2002). When maize kernels are damaged by insects, particularly moth larvae (Order *Lepidoptera*), they are much more susceptible to infection by mycotoxigenic fungi and subsequent mycotoxin contamination.

In North America, the European corn borer, *Ostrinia nubilalis* (Hübner) has been successfully managed through the strategy of broad deployment of Bt transgenes expressed under a very high dose *in planta*. For the first decade of Bt maize deployment, the dominant trait was based on expression of the insecticidal protein Cry1Ab, which is highly active against *O. nubilalis*. Very few borers survive in this system and populations have dropped to an historic low across the entire corn belt (Hutchison *et al.*, 2010), with no evidence for the development of resistance.

In contrast, other secondary Lepidopteran insects are moving into this space such as the corn earworm, *Helicoverpa zea* (Boddie) in the central corn belt of North America, the Western bean cutworm, *Striacosta albicosta* (Smith) in the more northern corn belt, and fall armyworm, *Spodoptera frugiperda* (J.E. Smith) in the southern corn belt. All these insects have been shown to influence mycotoxin levels in maize. The level of protection afforded by Cry1Ab-based transgenics to these secondary *Lepidopteran* insects is much less than for the European corn borer, and some do not work at all. Furthermore, fall armyworm has developed resistance to the Cry1F insecticidal protein in some of the extreme south parts of the corn belt (Storer *et al*., 2010). The widespread use of Bt maize and the subsequent drop in European corn borer populations in North America has probably contributed to lower average fumonisin levels over the last 15 years; however, that progress is threatened by the growing influence of other insects. Currently, biotechnology developers are stressing the use of insecticidal proteins other than Cry1Ab, or using newer traits together with Cry1Ab, in combinations that provide resistance to the full range of *Lepidopteran* pests. Fumonisin levels in these hybrids have been extremely low in field trials (Bowers *et al.*, 2013), and they show increased capacity to reduce aflatoxin levels as well.

The outlook for insect management in relation to mycotoxins in maize has two major facets: (1) the use of combinations of insect-resistance genes to manage multiple *Lepidopteran* pests (where transgenic maize is allowed); and (2) management of non-*Lepidopteran* insects not controlled by Bt transgenes (e.g. picnic beetle – *Glischrochilus quadrisignatus* [Say] (Dowd, 1998; J.C. Smith, personal communication), thrips – *Frankliniella* spp. (Parsons and Munkvold, 2010), and the brown marmorated stink bug – *Halyomorpha halys* [Stål], a new invasive pest in the north eastern corn belt (Hoebeke and Carter, 2003)) that also contribute to mycotoxin contamination, using insecticide applications and cultural practices such as planting date. Where transgenic maize is not allowed, the latter strategy must also be applied to manage *Lepidopteran* insects. A consortium of entomologists and pathologists is emerging to take a more holistic approach to characterising insect damage, its time of occurrence and severity relative to maize ear phenology and relating this to the development of

mycotoxins in the harvested grain. Without this relationship we cannot predict the minimum level of insect control that is required to have an economic impact on mycotoxin contamination in maize grain. In contrast, insect activity has little or no impact on mycotoxin risk in wheat and barley.

There is increasing evidence of important interactions between mycotoxigenic fungi and other plant pathogens. Maize plants with common smut infection (caused by *Ustilago maydis*) appear to be more vulnerable to infection by *A. flavus* and *F. verticillioides*, leading to increased levels of aflatoxins and fumonisins (H.K. Abbas, personal communication). Although common smut destroys many kernels on infected ears, the remaining kernels can become highly contaminated. Management tactics for common smut include genetic resistance and cultural practices related to fertility and successful pollination. Although resistance to common smut is not typically a high priority in maize hybrid development, it may be advisable to raise the level of effort in order to contribute to mycotoxin management. Some maize inbreds selected for resistance to *Fusarium* diseases also show elevated resistance to common smut (Mesterhazy *et al.,* 2012).

Abiotic factors influence all plant diseases, but these environmental effects can be multi-dimensional in the case of mycotoxigenic fungi, in the sense that conditions favouring infection and fungal growth are not necessarily identical to those that favour mycotoxin production. In general, the conditions that favour mycotoxin contamination for specific crop-fungus interactions, both pre-and post-harvest, are known in a qualitative way, and this information can serve as a framework for developing more quantitative models, which continue to evolve (e.g. Shah *et al.*, 2013). For example, in wheat, subpopulations of *F. graminearum* have been found to respond differently to heat and cold stress. Isolates that produce 3-ADON were more tolerant of extreme temperatures and produced higher trichothecene quantities than 15-ADON producing isolates *in vitro* (Vujanovic *et al.,* 2012). Further, understanding these nuances will inform predictions of potential shifts in the distribution and impact of mycotoxigenic fungi, due to changes in climate or production practices.

In wheat, fungicide application is a recognised and effective tool for managing mycotoxins, though the active ingredients, application timing, and application technology must be carefully selected. All available active ingredients fall under the single triazole group of fungicides, and have the same mode of action as sterol inhibitors. Reliance on a single group of fungicides puts management at great risk for the development of resistant *Fusarium* populations. So far, no resistance has been reported in North America and it was reported at the meeting that both the 15- and 3-ADON chemotypes of *F. graminearum* are equally sensitive to the available fungicides *in planta*. Currently available active ingredients have good efficacy against *Fusarium* species causing head blight, but there is significant room for improvement in field performance. Best results are usually obtained if genetic resistance is combined with carefully managed fungicide applications. The development of novel fungicidal compounds for commercial use is not specifically directed toward mycotoxigenic fungi, because of their relatively small market size in relation to the overall fungicide market.

Until recently, fungicide applications generally have not been successful for managing mycotoxins in maize, for several reasons. First, the economic benefits of controlling mycotoxigenic fungi in maize have been questionable. Second, a longer window of activity after application in maize is needed compared to wheat. Third, mycotoxigenic fungi in maize have diverse epidemiological characteristics, which complicate application timing and constrain the efficacy of single applications. Finally, traditional application technology has not proven effective in applying adequate doses of active ingredients in the right place at the right time, in a logistically manageable and cost effective manner. For fungicides to become a more successful tool in maize, it will likely require active ingredients with improved systemic and longer duration of activity, coupled with improved application technology that facilitates easier, more economical applications.

Some aspects of biological control approaches are attractive in maize and wheat. Many biological control agents have been tested in foliar applications to wheat, but their efficacy generally has been lower than that of fungicides. Approaches using microorganisms to break down sources of inoculum in wheat and maize fields has shown some promise (Gilbert and Haber, 2013). However, application may be limited to areas with diverse crop production and smaller, isolated maize and wheat fields. In North America, where large contiguous land areas are dedicated to maize or wheat production, airborne inoculum from other fields is believed to be adequate to cause outbreaks. Yet overall inoculum potential continually seems to rise with increasing levels of maize residue left in fields and reduced tillage. There were remarks at the meeting about the appearance of *Fusarium* perithecia on maize stalks as soon as three weeks after maize harvest, not previously observed by maize pathologists. Maize is by far the greatest contributor to the inoculum potential and there may be some merit in taking a fresh look at reducing this potential on a wider scale.

A method for suppressing infection by aflatoxigenic *A. flavus* strains, by field applications of atoxigenic strains of the same species, has been effective in research in North America and Africa. Application methods tested so far are ground-based, but there is work to be done on creating formulations of this material for aerial application. Although atoxigenic strains are not believed to pose a

risk for mutation restoring toxigenicity (e.g. Abbas *et al.,* 2011), the proposed use of atoxigenic *A. flavus* in aerial applications is controversial. Biological control agents may be valuable tools for FHB of wheat (e.g. Crane *et al.,* 2013). Progress would have to be made on their efficacy, shelf life, and ease of application in order for this approach to compete with the use of fungicides. Compared to other crops, approved biological controls might be more feasible than fungicide use for aflatoxin management in maize. However, no biological control methods appear to be highly effective for other mycotoxins in maize.

Post-harvest mycotoxin management typically includes sanitation practices, timely harvest, grain-handling to avoid damage, controlled temperature and moisture conditions in storage, and sorting procedures that remove infected kernels or seeds. Sorting and cleaning have been demonstrated to greatly reduce mycotoxins in grains and other products, but their practical application only seems financially feasible for food products, such as edible beans.

Grain treatment to suppress mycotoxigenic fungi in storage was one point of focus in the workshop discussion. Research in this area was aimed at grain treatments using less toxic, naturally occurring compounds other than traditional antifungal organic acids, such as propionic acid. Ozone has been shown to reduce populations of mycotoxigenic fungi in grain, reduce the accumulation of mycotoxins, and extend the safe storage life of grain (e.g. Aldred *et al.,* 2004) but there are issues that will constrain ozone application (K. Mylona and N. Magan, personal communication). Plant extracts such as Neem oil have antifungal properties and reduced mammalian toxicity compared to other alternatives. The capacity of these materials to suppress mycotoxin production in grain has not yet been demonstrated. It is important to understand how these treatments are affected by grain moisture and other environmental conditions, as well as the effects of the treatments on mycotoxin biosynthesis in addition to fungal growth and survival (Aldred *et al.,* 2008).

On-farm grain storage has increased dramatically in North America in the past 5 to 10 years. Farms are getting larger, and have moved up the grain handling chain, engaging in drying, conditioning, storage and trucking, capturing more value for their grain. Most grain stores also have grain-drying facilities. Most grain stores are corrugated galvanised steel bins and are very large (10 m wide and 30 m high), in clusters of 3 or more bins. Most have aerated floors and are serviced by a central dumping pit, and a bucket grain elevator, on a central leg with down spouts servicing each bin in the cluster. Most are emptied from the bottom. Little is known about the impact of this mode of storage and grain handling in the hands of primary producers on the level of mycotoxins. In the case of OTA, a great deal of the risk for contamination of the grain resides with the producer. This risk is new to the industry, and awareness is low. For example, it was shown that downspouts into grain bins coming from grain elevator legs, can serve as chimneys for warm damp air when grain is aerated to cool the grain down in winter on extremely cold days. The moisture can condense in these spouts and drip onto the grain surface that is often warm enough to support the growth of *P. verrucosum*, while the cooling front moves up through the grain mass over several days.

Producers are new to managing such large grain stores and several tools are emerging or required to assist. BINcast® is a platform that uses strategically placed sensors in the grain mass, coupled with outdoor weather data to monitor grain condition and to automate ventilation, all with remote access (Weather Innovations Consulting LP, Chatham, ON, Canada). The challenge for mycotoxins that accumulate during storage like OTA is that they tend to be produced in small pockets, under specific favourable conditions, and as grain stores become larger, these pockets are harder to find. Work is needed to understand how and where these pockets develop in the various types of stores and store management practices, so monitoring to ensure quality can be more strategic. Furthermore, as stored grain masses increase in size the ability to react to a problem becomes more difficult, again emphasising the need for proactive tactics. There is a good understanding among most grain store managers about maintaining grain quality macroscopically, but a poor understanding of the production of mycotoxins, such as OTA at the micro-level, and how this relates to the expanding scale of operations. In addition to the microenvironment at the top of the grain pile mentioned earlier, other microenvironments such as the grain/steel interface on the south-facing side of the bin during winter need to be identified and understood.

Assuring the quality of grain leaving a farm is important to the grain value chain. For most mycotoxins, especially those that are extremely heterogeneous and regulated at very low levels, these tools are very limited. Much work is needed to improve sampling and detection efficiencies. The use of $CO_2$ concentration as an indicator of grain quality is a thread that merits exploration (Maier *et al.,* 2010). From the data presented at the meeting, the development of specialised sensors such as the Mold Spy (Savory *et al.,* 2012) in grain systems also warrants consideration.

## 7. Mycotoxins in the feed and food production chain

Awareness of the impact of mycotoxins on industrial processing of grain maize has increased tremendously in the last 6 years, with the rapid expansion of the biofuel and bioproducts industry. In 2006, during the severe GER epidemic in the Great Lakes region of the corn belt, cash crop maize producers became acutely aware of the cost of

DON contamination when the ethanol industry routinely began refusing deliveries of grain maize exceeding 6 mg/kg DON. While it was widely understood that mycotoxins were process stable, and concentrated in the dried distiller's grains (DDGs) by a factor of 2.5 to 3 times (Schaafsma *et al.,* 2009), real and economic effects on ethanol production were noticed for the first time in 2006, when it was determined that the yeast became less efficient at these higher levels of toxin. Since 2006, grain loads are checked routinely for DON at the receiving point and those exceeding 5 to 6 mg/kg DON are rejected. Both the maize syrup and the ethanol industries have heightened awareness and surveillance in place for mycotoxins, expanding the direct impact of what was originally only serious in the swine industry, but is now a concern for most other end users of raw maize and DDGs. A better understanding of how mycotoxins affect industrial processes, where they are accessible to manipulation in these processes, and what tools can be developed to detoxify, divert or remove them are all targets worthy of pursuit. For example, some ethanol plants that recover water by centrifuge from stillage pass it through a short anaerobic digestion step before it is used again to make mash. Because DON is water soluble, this step may result in DON metabolism during digestion.

More reliable and informative sampling and more time-efficient and cost-effective analytical tools along the grain handling chain continue to be challenging needs warranting continuing research investment. For example, a swine producer will test incoming maize for mycotoxins, and, based on the test result, develop a feed ration, with, all too frequently, unexpected results, where high tests may result in little effect on swine performance, and low tests may result in poor performance. Work is needed to analyse the reasons for these unpredictable results, perhaps in the area of sampling, and analytical tools, and the presence and role of other related mycotoxin metabolites that are not measured by the current quick tests used in the industry, nor well understood.

The discussion concluded that the development of preventative strategies is key, as are appropriate incentives and tools to adopt best management practices linked to contract premiums, safeguarded by a crop insurance programme to protect the primary producer should the tools fail. A holistic approach engaging the entire value chain is necessary, perhaps developing a way to segregate a piece of the chain that is at highest risk, such as grains destined for infant foods, thereby relieving the pressure on the much larger, remaining portion of the chain.

Management of mycotoxins in the value chain is centred on health impact, the regulations that are in place and the surveillance that goes with them. Health Canada is working toward more targeted surveys of mycotoxin levels in domestic and imported foods. High analytical costs curtail the possible breadth and scope of surveillance, but targeting certain cereal-derived products that may contain OTA may inform how regulations are interpreted, applied and enforced in a more targeted manner. The use of weather-based tools to forecast the regional occurrence of mycotoxins has not been considered by regulators as a means to target surveillance more effectively and efficiently. Now that several mycotoxin forecasting models and platforms have been successful, there are opportunities to exploit these and develop more. These forecasting tools require long-term commitment and investment and their sustainability relies on consolidated effort and delivery. Even though the profit margins are very low, the private sector has the best record of sustaining the delivery of these tools, and government support would broaden their utility and accessibility.

Some mycotoxins, such as OTA, are particularly challenging to manage, predict and detect post-harvest/storage. In these cases prevention is critical. While best management practices can be developed that will greatly reduce the presence of OTA in grain leaving the farm store, their adoption is restricted by economics. If the regulation of OTA effectively applies to a small segment of the grain food chain, such as the infant food market, segregating the streams of raw grain from the outset into one with intense adoption of Grains Best Management Practice, traceability and HACCP may be possible. Grain producers, infant food manufactures and perhaps crop insurance providers could work out the incentives and catchments to fuel a segregated value chain, while the rest of the commodity value chain carries on in the open market.

## 8. Mycotoxin binders and treatments in animal feeds

There is little regulatory experience with the use of mycotoxin binders or bacteria that degrade mycotoxin in the guts of domestic animals. One exception is the use of calcium montmorillonite clays to reduce systemic exposure to aflatoxin. These clay products have been studied extensively for many years in domestic animals and have approval from the US Food and Drug Administration and the European Food Safety Agency (e.g. Aquilina *et al.,* 2012) as treatments to prevent aflatoxicosis. The approved clay has a high affinity and capacity to bind aflatoxin by chelation and cation exchange (Phillips *et al.,* 2002). The use of aflatoxin exposure biomarkers to assess the value of treated and untreated feed demonstrated significant toxin reductions and improved animal performance. These clays have also been tested for use in people who suffer chronic exposure to aflatoxin. Clays have been used as medicine for diarrhoea, wound healing, skin infection and other ailments, as well as binding agents for toxins. The ingestion of clay (geophagy) has been observed for centuries and across all continents and has ancient origins (Young *et*

*al.,* 2011). Trials have been conducted on populations in Ghana that showed the use of a modest amount of clay in the diet reduced serum aflatoxin adducts without affecting vitamin levels (Phillips *et al.,* 2008). Recently, reductions in systemic exposure to fumonisins in humans and rats have been demonstrated with these clays (Robinson *et al.,* 2012).

As each mycotoxin was chemically characterised, fungi as well as aerobic and anaerobic bacteria were found that could degrade the toxin (Karlovsky, 1999). In the past 5 years, investigators and companies have focused on the use of yeast products (various glucans) and microbials and enzyme products (Schatzmayr *et al.,* 2006). The glucans have a long history of safe use, but their efficacy as agents to ameliorate systemic exposure to mycotoxins is generally poor in independent tests (Fruhauf *et al.,* 2012; Kolosova and Stroka, 2011). With a long history of safe use, these products may prove to provide economic benefits to animal production (Jard *et al.,* 2011). The use of microbials and enzymes require toxicology and other safety studies for approval in Europe (there is as yet no defined pathway for approval in the US or Canada).

Treatment efficacy is increasingly being assessed by directly measuring bioavailability and effects on the animal, particularly on cells and tissues of the intestine. Aside from the glucans, microbes and enzymes, some substances that do not directly interact with mycotoxins (pharmacologic, antioxidant, immunostimulatory agents) may affect the toxicity of mycotoxins. In addition, the possibility of side effects, such as altered nutrient or drug uptake, must be considered. Recent studies have shown that the use of clays and glucomannan in swine diets affects the bioavailability of a veterinary drug (Devreese *et al.,* 2012; Goossens *et al.,* 2012).

There are some studies where additives have shown benefits under experimental conditions. The addition of glucomannan to the diet reduced immune suppression in pigs exposed to T-2 toxin or aflatoxin (Meissonnier *et al.,* 2009). In swine, dietary fumonisin and DON affects the levels of mediators of inflammation in the spleen (interleukin (IL)-6, IL-8, IL-1β, macrophage inhibitory protein β; Bracarense *et al.,* 2012). This model has been used to test the impact of additive effects. The addition of a mycotoxin-detoxifying agent resulted in a reduced impact on the chemokine indicators used (Grenier *et al.,* 2012).

In the USA and Canada, the regulation of feeds and feed additives is motivated by consumer safety and animal welfare considerations. The first issue is always the nature of the claim being made. If the additive or agent binds or affects the mycotoxin in the feed, it is regulated as a feed. If the action of the additive primarily occurs in the gut then normally this is regulated as a drug which in Canada requires both the Canadian Food Inspection Agency and Health Canada to participate in the review process. In the United States, the functions are both housed within the US Food and Drug Administration. Regardless of administrative process, the standards of evidence required are similar to those for any feed additive (e.g. dried distillers grains with solubles (DDGS)) or veterinary medicine. These studies must be performed using the common law requirements for all scientific data. That is, they must be conducted by qualified and experienced research personnel, using generally accepted methods, the data must be amenable to rigorous statistical analysis as appropriate and carried out under conditions similar to those in the USA or Canada. Under Canadian law, there is a requirement that efficacy be demonstrated. If the results of these studies are not published in good quality peer-reviewed journals, copies of the raw data and printouts of statistical analysis are required for review by the Canadian Food Inspection Agency. Each label claim must be supported by adequate research data (CFIA, 2012). The lack of an approved claim in Canada means that there is no experience with a completed file. Discussions in the workshop considered a number of recent findings. Purified glucomannan or glucomannan-containing yeasts have been investigated as mycotoxin binders. The efficacy of these in ameliorating the effects of DON was studied in *in vitro* and in chickens (Devreese *et al.,* 2012). *In vitro* assays using intestinal porcine epithelial cells (IPEC-J2) exposed to DON with or without active carbon or glucomannan suggested that active carbon was very effective and glucomannan partially effective in eliminating the cytotoxic effect of DON on cells and inhibiting its passage through the monolayer. An oral bolus containing DON with or without activated carbon or glucomannan was administered to chickens. In animals receiving active carbon, the plasma toxin concentration was below the limit of quantification while the plasma concentrations of toxin in animals receiving only DON and DON + glucomannan were not significantly different. These results indicate that the *in vitro* tests could be used as a tool for screening but results must be confirmed *in vivo*.

*S. cerevisiae* RC016 was able to bind and reduce bioavailability of several mycotoxins, including aflatoxin $B_1$, ZEA, OTA and fumonisin $B_1$, resulting in improved overall animal performance (S.N. Chulze, personal communication). The yeast could also co-aggregate with animal pathogens, playing a role in enhancing animal health. As yeasts are frequently fed to livestock as a good source of protein plus their probiotic effects, use of yeasts as mycotoxin binders should have more advantages compared to other mycotoxin binders. Research on understanding the binding mechanisms and on developing technologies to enhance binding efficacy and optimise mass production of yeasts should further improve the applications of yeasts as mycotoxin binders. However, like other mycotoxin binders, the applications of the yeasts in livestock also face the challenge of debinding after ingestion.

Detoxification by biotransformation results in preferably irreversible structural changes of mycotoxin molecules and is particularly useful for detoxifying mycotoxins for which there are no effective binders available, e.g. trichothecenes. Biotransformations can be catalysed by living microorganisms and their bioproducts such as enzymes, and can be achieved in animal intestines when applied as probiotics and feed additives or during feed-making procedures when used to treat contaminated raw materials. Before a biotransformation system can be used for mycotoxin detoxification, two fundamental issues need to be addressed – the toxicity of transformed products and the safety of the microorganisms used. A recent study discovered that the strain of *Devosia* sp. 17E, isolated from soil, could effectively transform DON into its stereoisomer, 3-epi-DON. The toxicity of 3-epi-DON has been evaluated in both *in vitro* and *in vivo* bioassays, and it has been proven that 3-epi-DON is much less toxic than DON (J. He, personal communication). Biotransformation reactions may involve multiple steps. For example, hydrolysed ZEA (HZEA) forms an intermediate during the transformation of ZEA to decarboxylated hydrolysed ZEA (DHZEA) by *Clonostachy rosea* (S. Fruhauf, personal communication). However, identifying the intermediate(s) can be a great challenge; one of the two intermediates formed during DON to 3-epi-DON transformation is still unidentified (J. He, personal communication).

*Bacillus subtilis* ANSB060 isolated from catfish is able to degrade aflatoxins $B_1$, $M_1$ and $G_1$ (Ma *et al.,* 2012). However, the bacterial isolate also showed antimicrobial activity towards several animal pathogens. Microorganisms with antagonistic activity can be a double-edged sword; in the animal intestine, they may inhibit pathogens but also interfere with normal microflora. Further studies are necessary to determine the antagonistic specificity of the strain before its widespread application (J. Yu, personal communication).

Mycotoxin detoxifying microorganisms are generally specific to particular mycotoxins. This is not the case with oxidative enzymes from the fungus *Trametes versicolor*. These have been shown to degrade aflatoxin (Zjalic *et al.,* 2006). Recent experiments demonstrate that the enzymes were able to degrade 55, 95 and 100% of aflatoxin $B_1$, DON and OTA, respectively, in solution. These three mycotoxins have considerably different chemical structures (C. Fanelli, personal communication).

Mycotoxin detoxifying enzymes can be identified directly through protein purification. An extracellular mycobacteria aflatoxins degradation enzyme produced by *Myxococcus fulvus* ANSM068 has been prepared and characterised (Ma *et al.,* 2012). The enzyme preparation showed high activity in degrading aflatoxins $B_1$, $G_1$ and $M_1$ in solution under a wide range of temperatures and pH values, a promising enzyme for the control of mycotoxins during food and feed processing.

Genes responsible for mycotoxin-detoxifying enzyme(s) can be identified using various genetic and genomic approaches. The gene(s) involved in the degradation pathway of ZEA by *Trichosporon mycotoxinivorans* (Vekiru *et al.,* 2010) were identified by obtaining mutants unable to degrade this toxin and sequencing both wild-type and mutant. Once the gene(s) are identified, they can be cloned into an expression host to produce the enzyme(s), and the enzyme yield can be elevated by increasing copies of the gene in the host (Hartinger and Moll, 2011). *FumD* encodes a carboxylesterase in *Sphingopyxis* sp. involved in the first step of fumonisin degradation. Recombinant *FumD* was heterologously expressed in the yeast *Pichia pastori* (M. Aleschko, personal communication). Heterologous gene expression can be an effective tool for large-scale production of mycotoxin-detoxifying enzymes.

## 9. Conclusions and recommendations

Jeffery W. Bentley wrote 'What farmers don't know can't help them ...' This is especially true in relation to the problem of mycotoxin contamination and exposure in different commodities and populations. To be effective, researchers must ensure that information and knowledge generated and gained through research goes beyond the collection of data, to reach the target groups. The researcher must also know what, how, where, when, and to whom the information should be communicated. In this way, research can claim to do more than produce a paper; it can achieve a purpose. For research to matter, it must be heard – and understood – by people in a position to bring about change. The way to make it heard, understood, and acted on, is effective knowledge translation, in formats that are easily understood by the target groups.

The cost of compliance with mycotoxin regulations in commodities, especially bulk commodities such as small grains and maize, is large but more importantly invisible to regulatory officials and other decision makers. These costs include sampling and analytical costs but also the larger costs of embargoed shipments of grain or grain held at country elevators as well as the cost of purchasing replacement grain. The cost to the food industry of destroying non-compliant product, particularly when facing a recall, is very large and includes public loss of brand confidence. A less obvious cost is the potential for a grain-producing region to be seen as an unreliable supplier of quality grain. This affects the producers, millers and shippers but also forces the food company to build an alternative supply chain thus increasing the costs of the raw material. These costs need to be considered when making regulatory decisions and emphasise the need to improve

methods to manage recurrent problems with particular mycotoxins.

For DON, science investments in plant breeding and agronomy have made a considerable difference in helping to manage this toxin in risk areas of North America. While FHB is a problem in many areas, the regions around the Great Lakes and in the Red River Valley area on both sides of the USA-Canada border are at most risk. The private and public sector programmes have resulted in improved cultivars of some wheat classes, however, little progress has been made in developing high-yielding maize hybrids with consistently adequate tolerance levels. The demand for higher yields to meet rising grain production costs continues to be a barrier to adoption of genetic resistance, because genetic resistance is polygenic and frequently comes at the cost of grain yield and composition. Because wheat is largely a food crop, the value chain has paid much more attention with greater success. Maize is a feed commodity, where yield is king, and the grain-handling trade profits from conditioning grain up the value chain. Yet, as the pressures mount to manage mycotoxin levels in maize because of the heightened awareness of their impact on industrial processes and restricted uses for industrial by-products, investment in research and education in hybrid resistance and selection, insect management, mycotoxin forecasting and fungicide application must continue. As we learn about the impacts of management efforts *in planta* on mycotoxin metabolites and we improve our ability to detect and quantify these, we must also invest in understanding their place in their overall toxicological and economic impact. We cannot afford to create new masked problems while apparently solving others.

Investments in fungal genomics research in the most well studied fungus, *A. flavus*, resulted in much surprising information on the ecology of this fungus but more importantly on its interactions with affected crop plants, mainly maize. The lesson that can be drawn from this is that investments in genomics of *F. graminearum* and *F. verticillioides* might well yield a similar payoff after more years of research. For the more saprophytic species, such as the ochratoxin-producing species of *Aspergillus* and *P. verrucosum*, the major need remains taxonomic clarity and identification tools that can be used by researchers without sequencing facilities. The maintenance of skilled researchers in fungal ecology and biosystematics as part of the research portfolio was judged essential. As climates and agronomic practices change, investment in research and surveillance to keep pace with concurrent shifts between and within fungal species is important, because of the clear evidence that such shifts may lead to more aggressive and higher toxin producing strains.

Several approaches to integrated mycotoxin management emerged from the discussion. Most of these approaches are not new, but perhaps the emphasis may need to shift based on what we know today. These are summarised as follows and are not arranged in any particular order:

1. *Managing inoculum potential*. Several cases of managing inoculum potential through sanitation, altered production practice and competitive exclusion have been shown to be successful, and merit further development. For example, lodged wheat, and tramping wheat during fungicide spraying may contribute to *Penicillium* inoculum in stored wheat grain; and success in displacing toxigenic *Aspergillus* strains with non-toxigenic ones may be an approach to follow in other systems. There are policy, logistic, environmental and health issues to overcome when developing these approaches, which need concomitant attention as they progress.
2. *Genetic control*. Genetic control continues to be the goal but achieving this has proven to be a challenge. Success in some crops against some plant pathogens has been much better than in others. Often the science outpaces policy and business, with economic and logistical barriers preventing adoption. Incentives to adopt useful technology also tend not to be considered while the technology is being developed. Nevertheless, genetic resistance has proven to be the most cost-effective management practice for many plant diseases, and it must continue to be emphasised for those diseases associated with mycotoxins. If mycotoxin monitoring is pushed up the value chain to the farm level, this may alter the balance of cultivar selection priorities and create incentives for farmers to favour more resistant genotypes, even at the expense of some yield potential.
3. *Insect control*. Managing crop injury caused by other pests is a broadly effective tool for managing mycotoxin contamination, particularly in maize. The impact of type and timing of injury and how this affects the accumulation of mycotoxins merits further study for major insect pests, but also for emerging insect species. The degree of control required for these pests of maize to materially reduce mycotoxin contamination needs to be understood. Neither insecticide nor the genetic approach has so far provided 100% control.
4. *Fungicides*. Fungicide use in mycotoxin management has been a challenging strategy, with good success in some crop/pathogen systems. Fungicides can be successfully employed for FHB suppression when combined with host genetics, and careful application and timing. The level of success in wheat suggests that exploring this avenue in other crop diseases with new fungicides is warranted. Only one class of fungicides, the triazoles, has been commercialised for use in mycotoxin management. This group of fungicides is characterised as being prone to the development of fungal resistance. We must be vigilant when exploring new avenues of chemical control and developing resistance management strategies to protect the tools we currently employ. If grain producers could be assured a consistent level of control of greater than

80%, along with a yield benefit greater than or equal to the cost of application, adoption of this technology would be swift. Biological controls applied as pesticides have met with limited success, but still warrant pursuit.

5. *Forecasting*. Mycotoxin contamination is dynamic during storage and mainly affected by environment, in particular water potential. There is tremendous potential in forecasting, identifying and solving condensation issues in modern grain stores for mitigating mycotoxin contamination in storage. Efforts to predict toxins in pre-harvest and in storage remain at variable stages of development. The most commonly used tools are for DON and are most successful at judging the value of applying fungicides at a time when the plant is most vulnerable to infection. The further development of these tools depends on their integration with the value chain and traceability measures. To achieve this, the idea of identifying an insurance benefit for following best practice and a price premium was discussed. In the case of recurrent and intractable low level contamination, this may be most beneficial for the public good.
6. *Analytical tools and sampling*. As regulations for mycotoxins expand in North America, integrated strategies that guide monitoring of susceptible commodities early in the value chain were considered ideal approaches. There are many areas where analytical and sampling methods need to be developed – or refined – in order to satisfy analytical needs along the entire grain value chain. Detection and management early in the value chain requires procedures that do not rely on complicated extraction methods and organic solvents; such commercially available test kits do not currently exist. Appropriate and efficient sampling and sample processing are also required to produce reliable results, particularly at the beginning of the value chain. User-friendly sampling methods are not generally available (or understood) by those charged with taking samples at the primary production end of the value chain. Sampling protocols for mycotoxins whose distribution in commodities are extremely heterogeneous, such as OTA in cereal grain, need to be developed and tailored to work at different steps along the value chain. The cost/benefit of sampling and testing for mycotoxins must also be considered; especially when preventing fungal growth and mycotoxin production may be more cost effective and easier to implement.
7. *Feed additives*. Additives to reduce the impact of toxins in grain that still meet regulatory guidelines require further study. In North America, these have not been given regulatory approval, thus information to assure reasonable certainty of no harm for meat, milk and egg products remains a research need. Investments in toxicology research for aflatoxins, fumonisins and DON have benefited agriculture by producing reasonable harmony in regulatory agencies on appropriate regulations or guidelines. In contrast, studies on the toxicity of OTA remain far from complete.
8. *Biomarkers*. Biomarkers of exposure are needed to strengthen relationships between human disease and exposure but also to more critically evaluate whether the proxy exposure estimates from food samples are reliable. Such studies have been conducted in the UK for a number of toxins and have been valuable (Turner *et al.,* 2012).
9. *Toxicology*. Finally, Health Canada's proposals to regulate OTA in Canada are having a significant impact on the grain sector in North America. As noted, there is no agreement in regulatory agencies in North and South America on the hazard level of this toxin. The European hazard assessment is different from that of the WHO. The application of ochratoxin guidelines in Europe is substantially different than in Canada and the USA because the scale of production and the scope of the supply chain are much greater in North America. It appears that further investments in toxicology research are needed to reduce safety factors. In addition, in Canadian (and USA) wheat-producing areas, the conditions leading to the formation of ochratoxin in storage are not understood well enough to manage it, except by exclusion when detected in the end product. Speakers from the farm, miller, and food sectors all expressed the need to be more involved in the regulatory process, especially in the risk/benefit discussion.

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