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Development of a novel and effective postharvest decision support system (DSS) for stored cereals to minimise mould spoilage and mycotoxins in food

Magan Centre of Applied Mycology, School of Environment and AgriFood

PhD in Environment and AgriFood

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Academic Year: 2020 - 2024

Supervisors: Professor Angel Medina-Vaya, Professor Rudolf Krska, Dr Julie Meneely (retired) and Professor Naresh Magan[†]

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This thesis is submitted in partial fulfilment of the requirements for
the degree of PhD

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I declare that:

- the thesis submitted has been written by me alone.
- the thesis submitted has not been previously submitted to this university or any other.
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Abstract

Cereal grains are widely consumed for their nutritional value as food and feed, and are essential in the food supply chain. However, changing climatic conditions have made these crops increasingly susceptible to fungal attacks, elevating the risk of contamination by mycotoxins—often referred to as "invisible mould poison." This can threaten grain safety and quality, posing health risks to humans and animals, and contributing to food insecurity and economic instability.

This thesis examines the effects of different abiotic factors (water activity- a_w and temperature) on the ratios of regulated and conjugated mycotoxin concentrations in naturally contaminated and irradiated wheat grains inoculated with *Fusarium graminearum*. Contaminated samples were analysed with Liquid Chromatography Tandem Mass Spectrometry. Deoxynivalenol-3-glucoside concentrations were significantly different from its precursor deoxynivalenol at 0.93 a_w (22% moisture content- MC) at 25 °C in the naturally contaminated wheat with a ratio proportion of 56:44, respectively.

This research further investigates the effects of different a_w and temperature on CO₂ production, fungal growth, and mycotoxin contamination in mini-silos of grains. It hypothesizes an integrated sensing approach (combining CO₂, temperature, and relative humidity measurements) as a decision support system (DSS) tool in real-time monitoring of CO₂ produced in stored grains would predict risks of mycotoxin contamination exceeding legislative limits.

Findings show that in naturally contaminated and inoculated (*Penicillium verrucosum* and *Fusarium langsethiae*) wheat and oat grains, respectively, an increase in a_w significantly increased the respiration rates (RR) and mycotoxin (ochratoxins, type A trichothecenes and their conjugate concentrations. Their legislative limits were exceeded at $\geq 0.80 a_w$ (16% MC) with $RR \geq 15 \mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$.

This research provides novel preliminary data for stored wheat and oats that can combine with other pre-harvest modules to develop a cost-effective DSS tool to improve grain storage management.

Keywords: wheat, oats, mycotoxins, water activity, CO₂ respiration rate, DSS, LCMS/MS.

Dedication

I dedicate this thesis to God Almighty, who made this PhD journey successful.

I also dedicate this thesis in memory of

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As he fondly asks. 'Are you winning'. Indeed, I HAVE WON.

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To my lovely husband –David Oluwakayode and
amazing children –Joanna and Esther Oluwakayode
for their love and huge support.

“The future belongs to those who
believe in the beauty of their
dreams.”

– Eleanor Roosevelt

“Don’t watch the clock; do what it
does. Keep going.”

– Sam Levenson

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Table of Contents

Academic integrity declaration.....	i
Abstract	ii
Dedication	iv
Acknowledgements	vi
List of Figures.....	xiii
List of Tables	xvi
List of Equations.....	xx
List of Abbreviations	xxi
1 Background and Motivation.....	1
1.1 Cereal grains	1
1.1.1 Composition and properties of cereal grains	1
1.1.2 Important cereals in the UK.....	1
1.1.3 Post-harvest losses of grains in the UK.....	6
1.2 Plants, fungi and mycotoxins interactions	8
1.2.1 Conjugated and Emerging Mycotoxins.....	8
1.3 Fungal genera and mycotoxins	11
1.3.1 <i>Fusarium</i> genus	12
1.3.2 <i>Penicillium</i> genus	13
1.3.3 Factors affecting mould growth and mycotoxin contamination in plants	14
1.4 Mycotoxin regulations, analysis and control.....	15
1.4.1 Mycotoxin regulations.....	15
1.4.2 Mycotoxins Analysis	16
1.4.3 Control strategies for mould and mycotoxins in stored grains.	17
1.5 Aim and Objectives	19
1.5.1 Novelty and linkage of project outputs.....	20
1.5.2 Outline of PhD Thesis	21
1.6 References	24
2 The initial mycobiota and mycotoxin contamination of grains harvested in different years in the UK.....	36
2.1 Introduction	36
2.2 Methodology	40
2.2.1 Grains.....	40
2.2.2 Moisture content (MC) analysis	41
2.2.3 Water activity (a_w) analysis.....	41
2.2.4 Fungal enumeration and isolation	42
2.2.5 Fungi isolation from cereals.	44
2.2.6 Statistical analysis	46
2.3 Results.....	47
2.3.1 Moisture content (MC) and water activity (a_w) analyses	47

2.3.2 Initial mycobiota of the grains.....	48
2.3.3 Preliminary screening for mycotoxigenicity using Ultraviolet light	56
2.3.4 Mycotoxins in grains.....	56
2.4 Discussion	60
2.5 Conclusions	62
2.6 References	64
3 Impact of environmental conditions on the concentrations of trichothecenes, their glucosides, and emerging <i>Fusarium</i> toxins in naturally contaminated, irradiated, and <i>Fusarium langsethiae</i> - inoculated oats.....	69
3.1 Introduction	71
3.2 Materials and Methods.....	73
3.2.1 Fungal isolates	73
3.2.2 Oat grains and moisture adsorption curve analysis.....	73
3.2.3 Grain inoculation	74
3.2.4 Mycotoxin analysis	75
3.2.5 Statistical Analyses	79
3.3 Results.....	80
3.3.1 Moisture adsorption curve (MAC) for natural and irradiated oats.	80
3.3.2 Fungal growth in stored oats	81
3.3.3 Method validation performance in the oat matrix.....	81
3.3.4 Initial mycotoxin concentrations of oats.....	83
3.3.5 Impact of a_w and temperature on the concentrations of T-2 toxin, HT-2 toxin, and HT-2-Glc in all oat treatments	83
3.3.6 The effect of the interactions of water activity, temperature, and treatments on the percentage concentration ratios of HT-2-Glucoside.	85
3.3.7 Impact of water activity, temperature, and treatments on DAS concentrations.....	87
3.3.8 Impact of a_w and temperature on DON and DON-3-G in both natural and irradiated oat grains with and without <i>Fusarium langsethiae</i> inoculation	89
3.3.9 Impact of a_w and temperature on the concentrations of other secondary metabolites and emerging mycotoxins in natural and irradiated oat grains with and without <i>Fusarium langsethiae</i> inoculation...	91
3.4 Discussion	93
3.5 Conclusion	96
3.6 References	98
4 The influence of different abiotic conditions on the concentrations of free and conjugated deoxynivalenol and zearalenone in stored wheat.	106
4.1 Introduction	108
4.2 Materials and Methodology.....	111
4.2.1 Fungal strain.	111
4.2.2 Wheat grains	111

4.2.3 Moisture adsorption curve analysis	111
4.2.4 Grain Inoculation	112
4.2.5 Mycotoxin analysis	112
4.2.6 Statistical analyses.....	116
4.3 Results.....	117
4.3.1 Moisture adsorption curve	117
4.3.2 Method validation performance in the wheat matrix.	117
4.3.3 The initial fungal isolation, deoxynivalenol, its glucoside DON-3-G, and ZEN concentrations of wheat grains before storage experiment.	119
4.3.4 Water activity and temperature impact on the concentrations of DON and DON-3-glucoside in naturally contaminated wheat grains with and without <i>Fusarium graminearum</i> inoculation.....	120
4.3.5 Water activity and temperature impact on the concentrations of DON and DON-3-glucoside in irradiated wheat grains with and without <i>Fusarium graminearum</i> inoculation.	120
4.3.6 Water activity and temperature impact on the concentrations of ZEN and its conjugates in natural wheat grains with and without <i>Fusarium graminearum</i> inoculation.	124
4.3.7 Water activity and temperature impact on the concentrations of ZEN and its conjugates in irradiated wheat grains with and without <i>Fusarium graminearum</i> inoculation.	124
4.3.8 Water activity and temperature impact on the concentrations of other secondary metabolites in natural wheat grains with and without <i>Fusarium graminearum</i> inoculation.	125
4.4 Discussion	128
4.5 Conclusion	133
4.6 References	134
5 The effect of the interactions of water activity, and temperature on OTA, OTB, and OT α produced by <i>Penicillium verrucosum</i> in a mini silo of natural and inoculated wheat using CO ₂ production as fungal activity sentinel.....	141
5.1 Introduction	143
5.2 Materials and Methodology	146
5.2.1 Wheat grains and moisture adsorption curve	146
5.2.2 Fungal strain and spores harvesting	146
5.2.3 Mini-silo CO ₂ sensing storage experiment.....	147
5.2.4 Fungal populations and isolations	148
5.2.5 Mycotoxin analysis	149
5.2.6 Statistical analyses.....	150
5.3 Results.....	151
5.3.1 Visual images of contaminated grains.....	151
5.3.2 Fungal populations in contaminated grains	151
5.3.3 CO ₂ production in contaminated wheat.	155

5.3.4 The influence of storage conditions on respiration rates in contaminated wheat	157
5.3.5 The influence of storage conditions on ochratoxin production in contaminated wheat.	158
5.3.6 Relationship between respiration rates and OTA in wheat.	160
5.4 Discussion	163
5.5 Conclusion	166
5.6 References	168
6 Real-time CO ₂ production monitoring in stored oats as an indicator of type A trichothecenes and ochratoxin A contamination under simulated environmental conditions.....	176
6.1 Introduction	178
6.2 Materials and Methodology	180
6.2.1 Oat grains and moisture adsorption curve analysis.....	180
6.2.2 Fungal strain and spores harvesting	181
6.2.3 Mini-silo CO ₂ sensing storage experiment.....	181
6.2.4 Fungal population and isolation.....	183
6.2.5 Mycotoxin analysis	184
6.2.6 Statistical analyses.....	187
6.3 RESULTS	188
6.3.1 Visual representation of contaminated oats.	188
6.3.2 Fungal populations and isolations in contaminated oats.	188
6.3.3 The influence of storage conditions on respiration rates in contaminated oats.....	191
6.3.4 Method validation performance in the oat matrix.....	193
6.3.5 The influence of the interactions of storage conditions on mycotoxin concentrations in contaminated oats.....	194
6.3.6 The relationship between respiration rates (RR) and type A trichothecenes and ochratoxin A in oats.	198
6.4 Discussion	202
6.5 Conclusion	207
6.6 References	208
7 Cost-benefit analysis of the integrated CO ₂ sensing approach as a decision support system (DSS) tool.....	215
7.1 Introduction	215
7.2 Methodology and Experimental Design	216
7.2.1 The cost analysis – case study	217
7.2.2 The integrated sensor system	217
7.2.3 The cost analysis for each integrated sensing approach.....	219
7.2.4 The benefits analysis:.....	219
7.3 Conclusion	225
7.4 References	226

8 General discussion, conclusions and recommendations	228
8.1 The initial mycobiota of UK cereal grains	228
8.2 Impact of storage conditions on mycotoxins in oats and wheat grains..	228
8.3 CO ₂ production in grain mini silos as an indicator of fungal activity and mycotoxin contamination	230
8.4 Could the CO ₂ DSS system be cost-effective?	231
8.5 Conclusions and contributions to knowledge	232
8.6 Recommendations for future research.....	233
8.7 References	235
Appendices.....	239
Appendix A Ethical approval letter	239
Appendix B Supplementary data for Chapter 2.....	248
Appendix C Supplementary data for Chapter 3	251
Appendix D Supplementary data for Chapter 4	255
Appendix E Supplementary data for Chapter 5.....	264
Appendix F Supplementary data for Chapter 6.....	267

List of Figures

Figure 1-1: Cereal grains (a) wheat (b) barley (c) oats (d) maize	2
Figure 1-2: <i>Fusarium</i> species (a) <i>F. graminearum</i> and (b) <i>F. langsethiae</i> on Malt Extract Agar (MEA+) media. +: chloramphenicol	12
Figure 1-3: <i>Penicillium</i> species (a) <i>P. chrysogenum</i> (b) <i>P. verrucosum</i> on Malt Extract Agar media.	13
Figure 1-4: The Interconnections of the project outputs and articles/manuscripts presented in this thesis.	21
Figure 2-1: Mean values of moisture content of cereal grains with standard error bars (n=3).	47
Figure 2-2: Mean values of water activity of cereal grains with standard error bars (n=3).	48
Figure 2-3: Pictorial representations of the initial microbiota in (a) wheat grains (b) barley grains (c) oat grains (d) maize grains for non-surface sterilised treatment on Malt Extract Agar	50
Figure 2-4: Frequency of isolation (%) of fungal diversity from naturally contaminated wheat grains on MEA+ and DG18+ media with surface sterilisation (SS) using 4% sodium hypochlorite (NaOCl) and non-surface sterilisation (NSS).	51
Figure 2-5: Frequency of isolation (%) of fungal diversity from naturally contaminated barley grains on MEA+ and DG18+ media with surface sterilisation (SS) using 4% sodium monochloride (NaOCl) and non-surface sterilisation (NSS).	52
Figure 2-6: Frequency of isolation (%) of fungal diversity from naturally contaminated oat grains on MEA+ and DG18+ media with surface sterilisation (SS) using 4% sodium monochloride (NaOCl) and non-surface sterilisation (NSS).	54
Figure 2-7: <i>Penicillium</i> species isolated from wheat grain under Ultraviolet (UV) light. Plate a represents the mycotoxigenic <i>Penicillium</i> (control) strain that fluoresce under UV light.	56
Figure 3-1: Moisture adsorption curves for 10g of (a) natural oats (d) irradiated oats grains.	80
Figure 3-2: Pictorial representation of fungal growth in stored oat grains at 20 °C (a) <i>Penicillium</i> at 0.95 a_w and (b) <i>Fusarium</i> at 0.98 a_w	81
Figure 3-3: HT-2-Glc concentration ratios (%) in all oat treatments and all storage conditions.	86
Figure 3-4: Impact of (a) water activity and (b) treatment on DAS concentrations (ng/g).	88

Figure 4-1: Moisture Adsorption Curves for 10 g of (a) natural wheat (d) irradiated wheat grains.	117
Figure 4-2: Pictorial representations of the Fungal isolations of the initial microbiota in (a) naturally contaminated wheat grains (b) irradiated wheat grains on Malt Extract Agar (MEA+).	119
Figure 4-3: The effect of water availability on the relative contamination with free, masked, and emerging mycotoxins in wheat.	132
Figure 5-1: Images showing contaminated wheat grains on day 20 at 15 °C with <i>Penicillium</i> growth at (a) 0.90 a_w and (b) 0.95 a_w	151
Figure 5-2: The respiration rates in contaminated wheat for the first 10 days at 0.90–0.95 a_w for both treatments at (a) 15 °C and (b) 20 °C with error bars (n=2).	156
Figure 5-3: The relationship between the respiration rates and concentrations of (a) OTA on day 10 (b) OTA on day 20 (c) OTB on day 10 (d) OTB on day 20 (e) OTalpha on day 10 and (f) OTalpha on day 20 at in contaminated wheat at different water activities.	162
Figure 6-1: Experimental design of the mini silo of CO ₂ sensing in stored oats.	181
Figure 6-2: Contaminated oats with mould spots after storing for 20 days at 15 °C.	188
Figure 6-3: The relationship between the respiration rates and the concentrations of (a) the sum of T-2+ HT-2+ HT-2-Glc on day 10 and (b) the sum of T-2+ HT-2+ HT-2-Glc on day 20 (c) OTA on day 10 and (d) OTA on day 20 in the contaminated oats at different water activities.	200
Figure 7-1: Grain silos with different decision support system tools.	217
Figure 7-2: The profit/ loss for different integrated sensing approaches over five years. T: temperature. RH: relative humidity. CO ₂ : Carbon dioxide.	223
Figure C-1: Pictorial representations of irradiated oat grains on Malt Extract Agar (MEA+)	251
Figure D-1: DON-3-Glc concentration ratios (%) in all wheat treatments and all storage conditions.	262
Figure D-2: ZEN-14-S concentration ratios (%) in all wheat treatments and all storage conditions.	263
Figure E-1: Mean values of CO ₂ respiration rates in naturally contaminated and <i>P. verrucosum</i> inoculated wheat at 15 and 20 °C for 20 days with standard error bars (n=2).	264

Figure E-2: Plot of the predicted against the known values of Ochratoxin A with residual plots	265
Figure F-1: Moisture Adsorption Curves for oat grains.....	267
Figure F-2: Pictorial representation of fungal populations/isolations in contaminated oats on MEA+ media at all water activity (a_w) levels at 15°C for Direct plating (DP) and Serial dilution (SD) methods.....	268
Figure F-3: Mean values of CO ₂ respiration rates in contaminated oats at 15 and 20 °C for 20 days with standard error bars (n=3).	269
Figure F-4: Plot of the predicted against the known values of T-2+HT-2+HT-2-Glc with residuals and normal Q-Q plots.....	271
Figure F-5: Plot of the predicted against the known values of OTA with residuals and normal Q-Q plots	272

List of Tables

Table 1-1: Table: Systematic definition of “modified mycotoxins”. Source Raycliff. et al. [46].	10
Table 1-2: Occurrence of conjugated and emerging mycotoxins in different food commodities. Source: Ekwomadu et al. [49].	10
Table 1-3: Maximum limits of UK-regulated mycotoxins in unprocessed cereal grains.	16
Table 2-1: Seasonal comparison between England and Northern Ireland.	38
Table 2-2: Seasonal mean air temperature and total precipitation in England and Northern Ireland from 2019-2021.	38
Table 2-3: Grains collected from different regions and harvested years in the United Kingdom.	40
Table 2-4: Optimised MS/MS parameters for the analysed mycotoxins, including precursor ions, product ions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP).	46
Table 2-5: Mean values ($n=3 \pm SD$) of the initial fungal populations ($\log_{10}$ CFUs/g) in the grains.	49
Table 2-6: The apparent recovery and limit of detection and quantification of the mycotoxins.	57
Table 2-7: Mycotoxin production and UV fluorescence by fungal strains isolated from grains.	57
Table 2-8: Mean values of the initial mycotoxin profile of grains	59
Table 3-1: Optimised MS/MS parameters for the analysed mycotoxins, including precursor ions, product ions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP).	77
Table 3-2: Mean values ($n = 75$) of the inter-day precision within-lab reproducibility (WLR) method validation for wheat grains.	82
Table 3-3: Influence of a_w and temperature on the concentrations of T-2, HT-2, and HT-2-Glc in oats.	84
Table 3-4: Influence of a_w and temperature on the concentrations of deoxynivalenol and DON-3-glucoside in oats.	90
Table 3-5: Influence of a_w and temperature on the concentrations of other secondary metabolites and emerging mycotoxins in oats.	92
Table 4-1: Optimised MS/MS parameters for the analysed mycotoxins, including precursor ions, product ions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP).	115

Table 4-2: Mean values (n=9) of the inter-day precision within lab reproducibility (WLR) method validation for wheat grains.	118
Table 4-3: Mean values of concentrations (ng/g) of Deoxynivalenol, its DON-3-glucoside and other derivatives in naturally contaminated wheat $\pm$ <i>F. graminearum</i> (n=4) at different storage conditions.	122
Table 4-4: Mean values of concentrations (ng/g) of DON and its bound DON-3-glucoside in irradiated wheat $\pm$ <i>F. graminearum</i> (n=4) at different storage conditions.	123
Table 4-5: Mean values of concentrations (ng/g) of ZEN and its glucosides in naturally contaminated wheat inoculated with and without <i>F. graminearum</i> (n=4) at different storage conditions.	126
Table 4-6: Mean values of concentrations (ng/g) of ZEN and its glucosides in irradiated wheat inoculated with and without <i>F. graminearum</i> (n=4) at different storage conditions.	127
Table 5-1: Mean values (n= 6 $\pm$ SE) of <i>Penicillium verrucosum</i> populations (log ₁₀ CFUs/G) in contaminated grains at all storage conditions.	153
Table 5-2: The effect of the interactions of storage conditions and their p values on <i>Penicillium</i> populations in contaminated wheat	154
Table 5-3: Frequency of isolation (%) of <i>Penicillium verrucosum</i> in contaminated grains at all storage conditions.	155
Table 5-4: Mean values (n=2) of respiration rates (μ g CO ₂ kg ⁻¹ h ⁻¹) in contaminated	157
Table 5-5: Mean values (n=2 $\pm$ S.D) of ochratoxins in contaminated wheat... ..	158
Table 6-1: Optimised MS/MS parameters for the analysed mycotoxins, including precursor ions, product ions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP).	185
Table 6-2: Mean values (n = 9 $\pm$ SD) of <i>Fusarium</i> and <i>Penicillium</i> populations (log ₁₀ CFUs/g) in contaminated grains at all storage conditions.	189
Table 6-3: Frequency of isolation (%) of <i>Fusarium</i> , <i>Penicillium</i> , and <i>Aspergillus</i> in contaminated grains at all storage conditions.	190
Table 6-4: Mean values (n=3 $\pm$ SD) of respiration rates (μ g CO ₂ kg ⁻¹ h ⁻¹)	192
Table 6-5: The effect of the interactions of storage conditions and their p values on respiration rates in contaminated oats	192
Table 6-6: Mean values (n = 48) of the inter-day precision within-lab reproducibility (WLR) method validation for oat grains.	193
Table 6-7: The mean values (n=4 $\pm$ SD) of the mycotoxin concentrations in the contaminated oats.	195

Table 6-8: The effect of the interactions of storage conditions and their p values on type A trichothecenes and ochratoxin A concentrations in contaminated oats.....	198
Table 7-1: Estimated expenses for each integrated sensing approach in grain silos for five years.....	221
Table 7-2: The cost-benefit analysis of a wheat silo with/without integrated sensors for five years.....	222
Table B-1: Average values ($n=3 \pm \text{SE}$) of moisture content and water activity of grains.....	248
Table B-2: Mean values ($n=3 \pm \text{SD}$) of the initial fungal populations ($\log_{10}$ CFUs/g) in the grains.....	249
Table B-3: Mean values ($n= 5 \pm \text{SD}$) of the initial fungal isolations in the grains	250
Table C-1: The measurement uncertainty for each analyte.....	251
Table C-2: Statistical differences in the concentrations of each trichothecene at all storage conditions using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.....	252
Table C-3: Statistical differences in the concentrations of trichothecenes at each water activity and temperature for all treatments using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method....	253
Table C-4: Influence of a_w x temperature on the concentrations of Diacetoxyscirpenol in oats.	254
Table C-5: Statistical differences in the concentrations of DON-3-G to DON at each water activity and temperature for all treatments using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.	254
Table D-1: Validation spiking levels in the wheat	255
Table D-2: The measurement uncertainty for each analyte.....	256
Table D-3: Mean values ($n=9$) of the Inter-Day Precision within lab reproducibility (WLR) method validation for wheat grains.....	257
Table D-4: Statistical differences in the concentrations of each analyte at all storage conditions using one-way ANOVA and nonparametric comparisons for each pair using Wilcoxon method.....	258
Table D-5: Statistical differences in the ratios of DON-3-G to DON at each water activity and temperature for all treatments using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method....	259

Table D-6: Statistical differences in the ratios of zearalenone (ZEN) to its conjugates at each water activity and temperature in the natural wheat control using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.....	259
Table D-7: Statistical differences in the ratios of zearalenone (ZEN) to its conjugates at each water activity and temperature in the natural wheat + <i>F. graminearum</i> using one-way ANOVA and nonparametric comparisons for each pair using Wilcoxon method.....	260
Table D-8: Statistical differences in the ratios of zearalenone (ZEN) to its conjugates at each water activity and temperature in the irradiated wheat control using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.....	260
Table D-9: Statistical differences in the ratios of zearalenone (ZEN) to its conjugates at each water activity and temperature in the irradiated wheat + <i>F. graminearum</i> using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.	261
Table E-1: Heat map of correlations between CO ₂ respiration rates, <i>Penicillium</i> populations, and ochratoxin concentrations in contaminated wheat for 0.90-0.95 a _w	266
Table F-1: Pearson Correlation coefficient (r) and p values of respiration rates and mycotoxin concentrations in contaminated oats.	270

List of Equations

(2-1)..... 41

(2-2)..... 44

(3-1)..... 78

(3-2)..... 78

(3-3)..... 78

(3-4)..... 85

(5-1)..... 147

(5-2)..... 148

(5-3)..... 149

(6-1)..... 182

(6-2)..... 183

(6-3)..... 184

(6-4)..... 186

(6-5)..... 186

(6-6)..... 186

List of Abbreviations

AcDON	Acetyldeoxynivaleno
ACN	Acetonitrile
AFB ₁ - M ₁	Aflatoxins B ₁ , B ₂ , G ₁ , G ₂ , M ₁
ANOVA	Analysis of Variance
a _w	Water Activity
BEA	Beauvericin
CBA	Cost-benefit analysis
CCA	Coconut Cream Agar
CFUs	Colony Forming Units
CO ₂	Carbon dioxide
DAS	Diacetoxyscirpenol
DG18+	Dichloran-glycerol media + chloramphenicol
DON	Deoxynivalenol
DON-3-G	Deoxynivalenol-3-Glucoside
DP	Direct Plating
DSS	Decision Support System
ENG	England
ENN	Enniatin
ESI	Electrospray ionization
FB ₁ - B ₃	Fumonisin B ₁ , B ₂ , B ₃
FHB	<i>Fusarium</i> Head Blight
FLO	<i>Fusarium langsethiae</i> inoculated oats
HAc	Acetic acid
HSD	Honestly Significant Difference
Hyd_FB ₁	Hydrolysed Fumonisin B ₁
IOC	Irradiated oat control
IOFL	Irradiated oat + <i>F. langsethiae</i>
IR	Irradiated
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
MC	Moisture content

MEA+	Malt Extract Agar + chloramphenicol
MON	Moniliformin
NA	Not applicable
NaOCl	Sodium hypochlorite
ND	Not detected
NI	Northern Ireland
NIV	Nivalenol
NO	Naturally contaminated oats
NOFL	Naturally contaminated oat + <i>F. langsethiae</i>
NSS	Non-Surface Sterilized
NW	Naturally contaminated wheat
NWFG	Natural wheat inoculated with <i>Fusarium graminearum</i>
OTA	Ochratoxin A
OTB	Ochratoxin B
OT α	Ochratoxin alpha
PHL	Post-harvest losses
RA	Absolute recovery
RE	Extraction recovery
RH	Relative humidity
RR	Respiration rate
SD	Standard deviation
SE	Standard error
sect	section
SS-	Surface Sterilized
SSE	Signal suppression/enhancement
UK	United Kingdom
US	United States
UV	Ultraviolet
WLR	Within-laboratory reproducibility
YES	Yeast Extract Sucrose
ZEN	Zearalenone
ZEN-14-G-	Zearalenone-14-Glucoside
ZEN-14-S	Zearalenone-14-Sulphate

ZEN-16-G- Zearalenone-16-Glucoside

1 Background and Motivation

1.1 Cereal grains

Cereal grains have been integral to the human diet for millennia, significantly influencing the development of human civilization. Globally, rice, wheat, and maize are essential staples, with sorghum and millet also playing a crucial role in providing daily sustenance for billions [1]. These grains contribute over 50 % of the world's daily caloric intake. The Food and Agriculture Organization forecasted global cereal production for July 2024 to be 2,854.2 million tonnes [2]. To align with consumer sensory preferences, most grains intended for human consumption are milled to remove the bran (pericarp) and germ [3].

1.1.1 Composition and properties of cereal grains

Cereal grains, including wheat, barley, oats, and maize, are essential for human nutrition due to their complex composition. They comprise 70–80% carbohydrates, 7.5–15% proteins, 1–4% lipids, fibres, vitamins, minerals (1.5–3% mainly phosphorus and calcium), and water [4]. Carbohydrates are primarily in the form of starch, and proteins contribute to the nutritional value and functional properties of food processing. Lipids are mainly concentrated in the germ, while fibres are predominantly found in the bran. These grains also contain B vitamins and essential minerals such as iron and magnesium. cereals have positive health effects [5]. Additionally, properly dried and stored grains typically have a water content of about 10-14% [6].

1.1.2 Important cereals in the UK

Figure 1-1 shows pictures of different types, shapes, and colours of the harvested grains mostly found in the UK and used in this research.



Sources: Figure (a) adapted from Redbook, [7], Figure (b & c): Laboratory pictures, Figure (d) adapted from Pinterest, [8].

Figure 1-1: Cereal grains (a) wheat (b) barley (c) oats (d) maize

1.1.2.1 Wheat

Wheat (*Triticum spp.*) is one of the most extensively grown and consumed cereal crops worldwide, serving as a staple food for about 40% of the global population [9]. Originating in the Fertile Crescent, wheat is now cultivated across diverse climates. In 2024, the Food and Agriculture Organization (FAO) reported that global wheat production reached about 789 million metric tons [2]. Major

producers of wheat include China (134 million metric tons), India (107 million metric tons), Russia (85 million metric tons), and the United States (49 million metric tons) [10]. Wheat is classified into durum, common, and club wheat, contributing essential amino acids, minerals, vitamins, beneficial phytochemicals, and dietary fibre to the human diet [11].

Global wheat consumption is just about its production value in metric tons, with significant usage in human food products such as bread, pasta, pastries, and animal feed. Consumption is expected to increase by approximately five million metric tons in the 2023/2024 trade year [12]. Due to the increasing human population and rising income levels leading to higher household consumption, it is anticipated that the demand for wheat will increase by an estimated 70% in the next decades (2020–2050) [13]. Additionally, external factors such as climate change and shifting patterns in consumer preferences towards starch-based foods are impacting the demand for wheat [9].

To meet future consumption needs, increasing wheat yields in the coming decades is a crucial goal for current agricultural policy. Achieving this will involve improving farm production systems, introducing new technology, developing better wheat varieties, identifying optimal crop rotation and diversification strategies, improving plant protection, and gaining continued support from stakeholders throughout the value chain, including technology providers, processors, manufacturers, and governments [14–16].

In the UK, wheat is the most significant cereal crop in terms of area planted and production volume. According to the Department for Environment, Food & Rural Affairs (DEFRA), the UK's wheat production was approximately 14.3 million metric tons in 2021 and over 15.5 million metric tons in 2022 [17]. The country primarily grows winter wheat, sown in autumn, and harvested the following summer. The UK consumes approximately 15.5 million metric tons of wheat annually [17]. However, this value varies in different years. Imported wheat grains are required when the production value is lower than the consumption value due to an increase in demand. A significant portion of wheat is used for milling into flour for bread, other baked goods, and livestock feed [17]. The adaptability, high

yields, and nutritional profile of wheat, along with its ease of harvesting, storage, transportation, and processing, have undoubtedly contributed to its widespread success.

1.1.2.2 Barley

Barley (*Hordeum vulgare* L.) is one of the oldest cultivated grains, with its origins in the Near East. It is versatile, used for animal feed, human consumption, and as a key ingredient in brewing and distilling industries; therefore, the spoilage of barley grains will negatively impact the brewing industry [18]. It ranks fourth in both quantities produced and in the area of cultivation of cereal crops in the world [19]. In the 2022/23 season, the global barley production was approximately 154 million metric tons. The largest barley producers are the European Union (EU) with 51.6 million tons, followed by Russia (21.5 million metric tons), Australia (14.1 million metric tons), and Canada (9.9 million metric tons). Global barley consumption is around 152 million metric tons of which 68% is used for animal feed and feed ingredients [20].

The barley head, also known as the spike, is composed of spikelets attached to the rachis in an alternating pattern. The outer layers of the barley kernel include a husk, which completely covers the grain; the pericarp (to which the husk is tightly joined in most species); the testa or seed coat, and the aleurone [18]. Barley grains are good sources of nutrients such as starch, proteins, lipids, dietary fibre, and B vitamins. They also have phytochemicals with antioxidant activities, such as polyphenols, vitamin E, and carotenoids, hence their use for human consumption [21].

The UK is a major producer of malting barley, with a production of about 7.1 million metric tons in 2021 [17]. Spring barley is more commonly grown than winter barley due to its suitability for malting. The annual consumption of barley in the UK is approximately 6.8 million metric tons, with widespread use in brewing, distilling industries, and animal feed [17].

1.1.2.3 Oats

Oats (*Avena sativa L.*) are primarily grown in temperate regions, with origins believed to be in the Fertile Crescent. Oats are the sixth most produced grain worldwide, following wheat, corn, rice, barley, and sorghum. Their shape is similar to barley with different types such as black, red, yellow, and white oats [22]. They are valued for their health benefits. They are rich in protein, fibre, calcium, and vitamins such as B, C, E, and K. They also contain amino acids and antioxidants, which can help reduce cholesterol and aid in weight loss by improving the body's lipid profile and breaking down fat [23].

The global oat production was about 26 million metric tons in 2021 with major producers such as Russia (4.5 million metric tons), Canada (4.2 million metric tons), Poland (1.3 million metric tons), and Finland (1.2 million metric tons). Oat consumption globally is roughly 25 million metric tons, with usage in food products such as oatmeal and animal feed [2].

Oat production in the UK has seen a resurgence due to increasing demand for healthy foods. The crop is well-suited to the UK's cool, wet climate, and is predominantly grown in Scotland and northern England. In 2022, the volume of harvested oat production was measured at approximately 1.0 million metric tons with a value of £301 million [24]. UK oats are valued for their high quality and are used in a variety of food products, including porridge and oat-based snacks. Additionally, oats are part of sustainable crop rotation practices.

1.1.2.4 Maize

Maize (*Zea mays L.*) originating from Central America serves as a staple food for large population countries such as America (with 40% production of global maize yield), China, Brazil, and Argentina. Maize is rich in B vitamins, thiamine, niacin, pantothenic acid (B5), and folate hence, an important source of food and feed

[25]. Maize is also significant for industrial uses, including biofuels and bioplastics [26]. Maize kernels exhibit a range of colours, including white, yellow, red, and black. Additionally, maize can be categorized by the size and composition of its endosperm, leading to varieties such as dent, flint, waxy, flour, sweet, pop, Indian, and pod corn. Another classification method focuses on the sweetness or sugar content, which varies with the variety and harvest time. Sweet maize is best consumed fresh, canned, or frozen promptly, as it does not store well and quickly becomes tough and starchy [27].

Maize production in the UK has increased in recent years, primarily driven by its use as a forage crop for livestock and as a feedstock for anaerobic digestion in bioenergy production. While the UK does not have the same extensive maize-growing areas as countries like the US or Brazil, it has adapted to grow varieties suitable for its cooler climate. Maize is typically grown in the south and east of England, where conditions are more favourable [28].

1.1.3 Post-harvest losses of grains in the UK

Pre-harvest grain losses occur before grains are harvested and are commonly due to diseases, pathogens, pests, abiotic stresses or weather conditions and improper agricultural practices [29]. To ensure high-quality grain, cereal processing from preharvest to storage involves a series of steps beginning with crop selection, soil preparation, and proper crop management practices like irrigation, fertilization, and pest control.

Timely harvesting, either manually or mechanically, is crucial to minimise losses. After harvesting, grains undergo threshing to separate them from the chaff, followed by cleaning, drying to safe moisture levels, and grading based on quality. Finally, cereals are stored in dry, pest-free conditions using bags, silos, or hermetic containers, with regular monitoring to preserve grain quality and prevent spoilage [29,30].

Post-harvest losses for cereals in developed countries, including the UK, are typically estimated between 1% and 5% of total production—significantly lower

than the 20% to 30% seen in developing countries due to infrastructure and handling challenges. According to the Food and Agriculture Organization (FAO), grain losses in countries like the UK are usually under 2%. However, this is still substantial given the large volumes produced. For example, with the UK's wheat production exceeding 14 million tonnes annually, this could mean losses of around 280,000 tonnes [31].

Waste and Resources Action Programme study [32] indicated that food waste, including cereals at the post-harvest stage, could result in losses worth around £1.4 billion annually, with grains comprising a large portion of these losses. Improper storage conditions, such as excessive moisture or pest infestations, can lead to 0.5% to 1.5% loss in grains like wheat and barley. Insects, fungi (such as *Fusarium*), and rodents contribute to an additional 0.3% to 1% of post-harvest losses, depending on how well the grain is stored and managed.

Controlling grain storage is crucial for maintaining grain quality, safety, and quantity. Spoilage in stored grains is promoted by moisture in improperly dried grains stored in silos, ingress of water into silo due to silo leakage, or changes in temperature in the storage environment influenced by weather conditions [33]. Some existing systems to monitor and control spoilage include efficient drying before storage, pest control, moisture and temperature monitoring, and aerating systems that use fans to circulate air through the grain mass to reduce moisture content and temperature or maintain uniform conditions. However, when the relative humidity is high such as we have in the UK, the drying of grains becomes slower, and spoilage becomes imminent [34,35].

Therefore, implementing good agricultural practices (GAP) during pre-harvest and proper storage post-harvest of cereal grains is crucial to maintaining their quality, preventing spoilage, and minimizing the risk of mycotoxin contamination [36].

1.2 Plants, fungi and mycotoxins interactions

The interaction between plants, fungi, and mycotoxins is a multifaceted relationship that significantly impacts crop health and food safety. Fungi such as *Fusarium*, *Aspergillus*, and *Penicillium* can infect plants under favourable conditions, which are often triggered by environmental stress or damage, leading to the production of mycotoxins like aflatoxins, fumonisins, and deoxynivalenol (DON) [37,38]. These toxins pose serious risks to human and animal health and can impair plant physiological processes, reduce yield, and compromise grain quality [38]. Some plants possess innate defence mechanisms that limit fungal colonisation or detoxify mycotoxins, but susceptibility varies widely across species and environmental conditions [39]. Understanding these interactions is key to developing integrated strategies for crop protection, including resistant cultivars, biological control agents, and improved post-harvest practices. Mycotoxins are naturally occurring toxic secondary metabolites produced by filamentous fungi, posing significant risks to human and animal health and causing economic instability [40]. Mycotoxins are a significant concern in cereals such as wheat, maize, barley, and oats.

1.2.1 Conjugated and Emerging Mycotoxins

It is important to note that mycotoxins can be present in food and feeds in their 'free' or 'modified/conjugated' forms. Conjugated mycotoxins are formed as part of plants' defence mechanism against infection by fungi. The plant metabolises some of the 'free' mycotoxins produced by the fungi through biotransformation to produce other forms of mycotoxins which are generally considered 'less toxic' but can revert to its precursor toxin in the gastrointestinal tracts of mammals [41]. The biotransformation processes mainly include hydroxylation, hydrolysis, oxidation, hydrogenation, sulfation, esterification, de-epoxidation, methylation, glycosylation, glucuronidation, demethylation, and deamination which are caused by either living organism or isolated enzymes [42].

Another growing area of concern in food safety is emerging mycotoxins. These are not widely studied or regulated as traditional mycotoxins, more research is needed to understand their full impact on human and animal health, as well as

their occurrence in the food supply. Examples of emerging mycotoxins commonly found in cereal grains include Enniatins, Beauvericin, Moniliformin, Fusaproliferin, and Sterigmatocystin [43–45].

According to Raycliff, et al. [46], “modified mycotoxins” suggests any modification of the basic chemical structure of mycotoxins, either by chemical or biological modifications. Biological modifications include Phase I metabolism involving hydrolysis or oxidation e.g aflatoxin B1 exo-8,9-epoxide. Phase II metabolisms are characterised by conjugation reactions by plants such as the formation of DON-3-Glc or ZEN-14-Glc or conjugation by fungi e.g ZEN-14-sulfate. The reactions in phase I do not always lead to components with decreased phytotoxicity compared to the original xenobiotic itself; in some cases, the metabolite is as toxic as the parent compound, and in others, there is even a considerable increase in toxicity [47].

Chemically modified mycotoxins can be classified as “thermally formed” and “non-thermally formed”. Thermal degradation reactions and thermal modifications occur during food and feed processing including baking, roasting, frying, or extruding. Example is fumonisin FB₁ which can react in a Maillard-type reaction with reducing sugars leading to N-(1-deoxy-Dfructos-1-yl) fumonisin B₁ and N-(carboxymethyl) fumonisin B₁ [48]. Examples for non-thermal modifications of mycotoxins are the formation of hydrolysed fumonisins (HFB₁) or the above-mentioned norDON A–C degradation products formed under alkaline conditions [48]. Table 1-1 below highlights the proposed definition of conjugated mycotoxins adapted from Raycliff et al. [46] while Table 1-2 shows the occurrence of conjugated mycotoxins in different food commodities, adapted from Ekwomadu et al. [49].

Table 1-1: Table: Systematic definition of “modified mycotoxins”. Source Raycliff. et al. [46].

1 st stage	2 nd stage	3 rd stage	4 th stage	Example
Free mycotoxins				DON, Aflatoxin B1, 3-acetyl-DON, 15-acetyl-DON
Matrix-associated mycotoxins	Complexes, physically dissolved or trapped Covalently bound			Fumonisin bound to starch, OTA- and DON-oligosaccharides Aflatoxin B1-epoxide
Modified mycotoxins	Biologically modified	Functionalised (phase 1– metabolites) Conjugated (phase 2 – metabolites)	Conjugated by plants (= masked according to ILSI) Conjugated by animals Conjugated by fungi	DON-3-glucoside DON-3/8/15-glucuronide, HT2-3/4-glucuronide ZEN-14-sulfate
	Chemically modified	Differently modified Thermally formed Non-thermally formed		Deepoxy-DON (=DOM-1) norDON A-C, N-carboxy-methyl-FB1, 14-(R)-OTA DON-sulfonate, norDON A-C (under alkaline conditions)

DON: Deoxynivalenol, OTA: Ochratoxin A, ILSI: International Life Science Institute.

Table 1-2: Occurrence of conjugated and emerging mycotoxins in different food commodities. Source: Ekwomadu et al. [49].

Mycotoxins	Food	% Positive samples	Range (µg/kg)	References
DON-3-G	Wheat	83.3	43-737	[50]
	Barley	29	43-277	[51]
	Maize	80	14-121	[52]
	Oats	87.1	<LOQ- 6600	[53]
ZEN-14-G	Wheat	6.7	<LOQ-0.6	[53]

Chapter 1

	Barley	17.6	<LOQ-9.6	[53]
	Maize	17	274	[54]
	Oats	3.2	<LOQ	[53]
ZEN-16-G	Wheat	6.7	<LOQ-2.8	[53]
	Barley	23.5	<LOQ	[53]
	Oats	58.1	<LOQ-7.9	[53]
ZEN-14-S	Wheat	33.3	11	[54]
	Barley	8.8	<LOQ-26.1	[53]
	Maize	17	51	[54]
	Oats	16.7	12	[54]
HT-2-Glc	Wheat	53	<LOQ-15.9	[53]
	Barley	94.4	0.6–162.8	[55]
	Oats	16.1	<LOQ-300	[53]
Enniatin B1	Maize	2	0.13-3	[49]
Moniliformin	Maize	63	<LOQ- 1130	[49]
Beauvericin	Maize	51	<LOQ- 142	[49]

1.3 Fungal genera and mycotoxins

Two common fungal genera colonising grains at pre-harvest and post-harvest, especially during storage and producing mycotoxins, are *Fusarium*, and *Penicillium* [56-58].

1.3.1 *Fusarium* genus

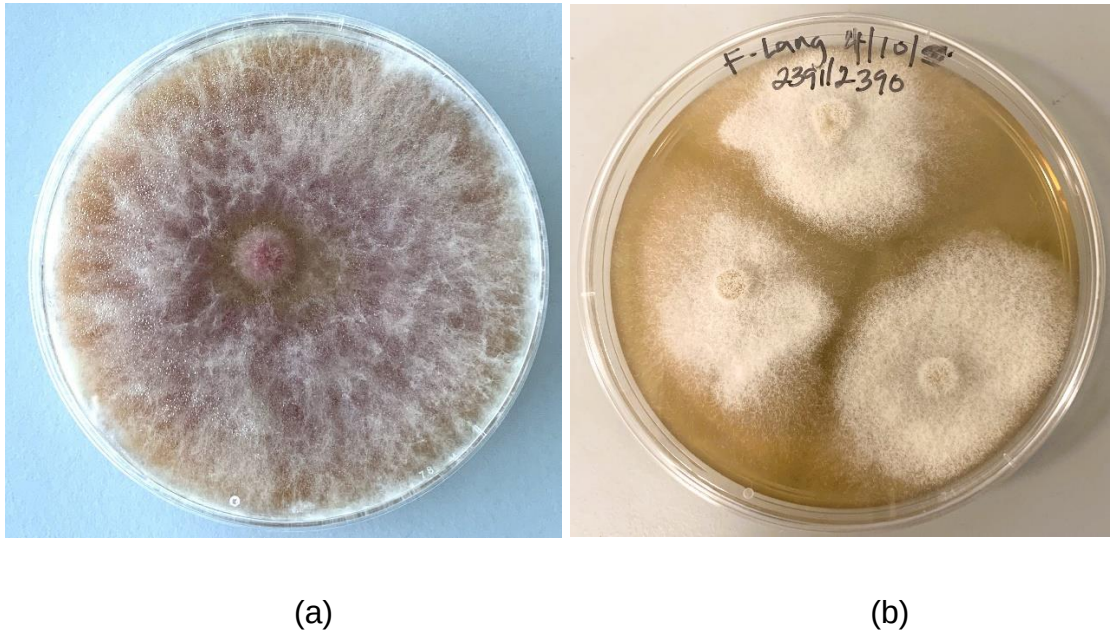


Figure 1-2: *Fusarium* species (a) *F. graminearum* and (b) *F. langsethiae* on Malt Extract Agar (MEA+) media. +: chloramphenicol

Fusarium species are the major cause of diseases such as *Fusarium* Head Blight (FHB) in wheat and ear rot in maize resulting in substantial yield loss and mycotoxin contamination, posing significant risks to food safety and human health [59,60] *Fusarium* species are commonly found in damp conditions [36,61]. Some *Fusarium* species and their associated mycotoxins include;

- *Fusarium graminearum* and *F. culmorum*: major causes of FHB in wheat and barley, producing deoxynivalenol (DON) and zearalenone (ZEN) [62,63].
 - DON is a vomitoxin, that causes vomiting in humans and animals. It is usually produced at water activity levels of 0.90 to 0.95 a_w .
 - ZEN is an estrogenic mycotoxin affecting reproductive health produced around 0.90–0.97 a_w [64-67].
- *Fusarium langsethiae*: a major producer of T-2 toxin and HT-2 toxins in oats.
 - T-2 /HT-2 toxins weaken the immune system, retard growth in exposed animals, and cause cytotoxicity in the human small

intestine [68]. These toxins are mostly produced at 0.90 to 0.98 a_w [69].

- *Fusarium verticillioides*: common in maize and associated with *Fumonisin* (B₁, B₂, and B₃) production at 0.93 to 0.95 a_w .
 - FB₁ is linked to esophageal cancer in humans, leukoencephalomalacia in horses and pulmonary edema in pigs [64,65].
- *Fusarium proliferatum*: found in maize and other cereals, producing fumonisins 0.93 to 0.95 a_w and fusaproliferin [70–72].

1.3.2 *Penicillium* genus

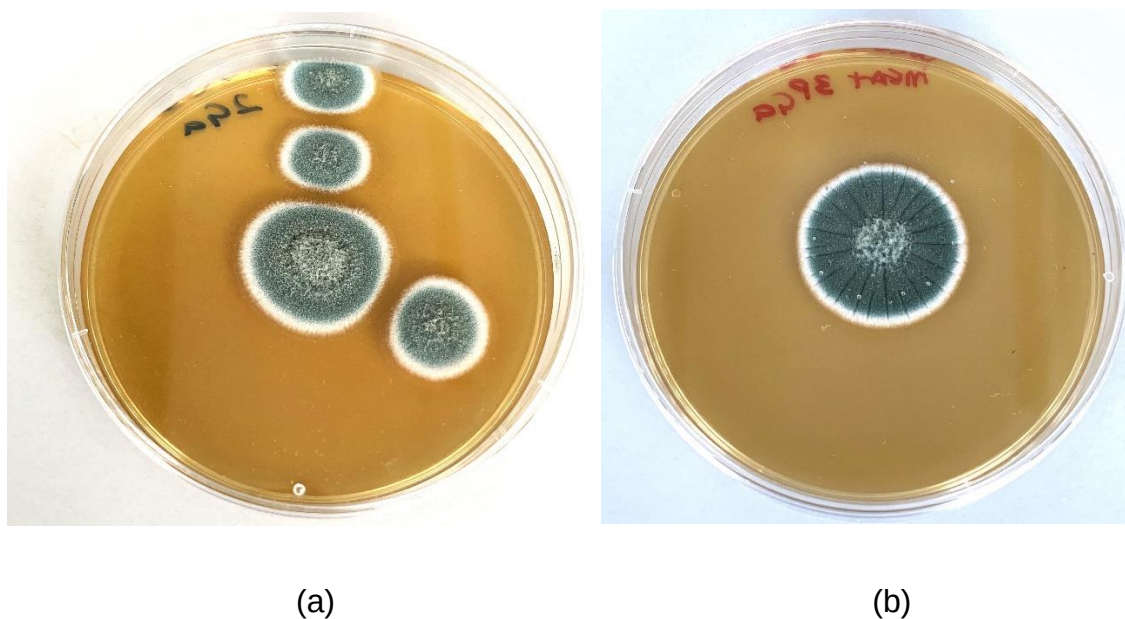


Figure 1-3: *Penicillium* species (a) *P. chrysogenum* (b) *P. verrucosum* on Malt Extract Agar media.

Penicillium is well known and distributed worldwide and has a significant economic impact on human life. Its primary role in nature is to break down organic

materials, and certain species can cause destructive rots as pre- and postharvest pathogens on food crops [73]. *Penicillium* species thrive in cool, damp conditions. *P. verrucosum*, for example, grows well at temperatures between 0–31 °C, with optimal growth at around 20 °C producing OTA around 0.80–0.85 a_w [74]. *Penicillium* species are known for producing mycotoxins such as ochratoxin A (OTA) and citrinin. Some species include;

- *Penicillium verrucosum*: produces ochratoxin A (OTA) in species such as wheat [75].
- *Penicillium expansum*: commonly associated with patulin production and can thrive well at $> 0.95 a_w$. It can grow well at temperatures as low as 0°C in cold-stored fruit.
 - Patulin is more frequently a problem in fruits than cereals [76].
 - Patulin production can occur at 0.88–0.99 a_w at 0–25 °C [77].
- *Penicillium citrinum*: produces citrinin, which can affect kidney function and has been implicated in nephrotoxicity and possibly carcinogenic.
 - Citrinin production generally occurs at 0.80–0.98 a_w at 25 and 30 °C, while *Penicillium citrinum* growth can occur at 0.83 a_w and at low temperatures, but citrinin production is more efficient at higher water activities [78].

1.3.3 Factors affecting mould growth and mycotoxin contamination in plants

The two major factors influencing fungal growth and mycotoxin contamination in stored grains are water activity and temperature [36,79].

1.3.3.1 Water activity

Water availability plays a crucial role in cereal grains' storage, preservation, and safety. Moisture content refers to the water in the grains, while water activity indicates the availability of free water for microbial growth and chemical reactions [80]. Lower water activity inhibits the growth of moulds, and other pathogens, the major factors contributing to grain spoilage and mycotoxin production.

Water activity is typically measured using hygrometers or water activity meters, which determine the relative humidity of air in equilibrium with the sample [81]. For effective storage of grains after harvest, reducing and maintaining water activity to $< 0.70 a_w$ or its moisture content equivalent of $<14\%$ is recommended. This inhibits mould growth, allowing extended storage without significant quality loss [82].

1.3.3.2 Temperature

Temperature is a key factor influencing mould growth and mycotoxin contamination in grains. Elevated temperatures, especially when combined with high humidity, create favourable conditions for fungi such as *Aspergillus* and *Fusarium* species to proliferate and produce mycotoxins [36, 83]. The optimal temperature for fungal growth varies but typically ranges between 20–30 °C. Storage in cooler conditions can inhibit fungal activity.

Other factors include prolonged storage duration, where grains are exposed to favourable conditions for fungal growth. Physical grain damage during shattering at harvest or improper handling can provide entry points for fungal infection. Poor storage hygiene, including contamination from previous grain batches, or grain handlers, can introduce and spread fungal spores.

1.4 Mycotoxin regulations, analysis and control

1.4.1 Mycotoxin regulations

To mitigate the risks of mycotoxins various countries and international organizations have established regulations and guidelines to control mycotoxin levels in food and feed. These regulations aim to ensure food safety and protect consumer health by setting maximum permissible levels of mycotoxins in various commodities. The European Union (EU) has some of the most comprehensive regulations on mycotoxins, detailed in Regulation (EU) 2023/915 [84] and its subsequent amended regulations; Regulation (EU) 2024/1022 [85] and Regulation (EU) 2024/1038 [86]. The EU sets strict maximum levels for various

mycotoxins in food products, including aflatoxins, ochratoxin A, patulin, DON, zearalenone, and fumonisins. These regulations cover multiple products, from cereals and nuts to baby foods. The EU also enforces stringent monitoring and reporting requirements to ensure compliance [84].

Following Brexit, the UK has rules and strict limits for aflatoxins, ochratoxin A, patulin, and *Fusarium* toxins in certain foods. The maximum levels (MLs) are established in the assimilated Regulation (EU) 1881/2006 for England and Wales and the Regulation (EU) 1881/2006 for Northern Ireland [87].

The Food Standards Agency (FSA) in England, Food Standards Scotland (FSS), the Food Standards Agency in Wales, and the Department of Agriculture, Environment and Rural Affairs (DAERA) in Northern Ireland oversee the enforcement of these regulations [88]. The maximum permitted limits of regulated toxins in unprocessed cereal grains are shown in Table 1-1 below [84–87].

Table 1-3: Maximum limits of UK-regulated mycotoxins in unprocessed cereal grains.

Mycotoxins	Maximum limits (µg/kg)
Aflatoxins (sum of B ₁ , B ₂ , G ₁ & G ₂)	4.0
Ochratoxin A	5.0
Zearalenone	350
Deoxynivalenol	1750
T-2 and HT-2 toxin	1250
Fumonisins (sum of B ₁ & B ₂)	4000

1.4.2 Mycotoxins Analysis

Various analytical methods have been developed and published to detect mycotoxins in foods and feeds. These methods include Enzyme-Linked Immunosorbent Assay (ELISA), which is simple, rapid, and cost-effective [891]

but not sensitive enough to detect very low levels of analytes, especially in complex biological samples [90]. Thin-layer chromatography is a rapid, easy-to-use, and cost-effective analytical technique. It is however limited in sensitivity with poor quantitative accuracy [91,92].

Advancements in analytical techniques have significantly improved the detection and quantification of mycotoxins in various matrices. Common methods include High-Performance Liquid Chromatography (HPLC), often coupled with mass spectrometry (MS) or fluorescence detection, widely used for its sensitivity and specificity [93]. Liquid Chromatography-Mass Spectrometry (LC-MS/MS) combines the physical separation capabilities of liquid chromatography (LC) with the mass analysis capabilities of mass spectrometry (MS). As an advantage over other analytical methods, it offers high sensitivity and accuracy, allowing for the simultaneous detection of multiple mycotoxins even at low levels of analytes [94,95]. However, the advanced chromatographic methods are more expensive with costs covering the initial setup and maintenance of LC-MS/MS systems, the costs of equipment, consumables, and skilled personnel [96]. It requires more time for method development and sample preparation. Complex biological matrices analysed with LC-MS/MS can lead to ion suppression or enhancement (matrix effect), affecting the accuracy and reproducibility of the analysis [97]. Data generated by LC-MS/MS can be complex and require sophisticated software and expertise to interpret correctly, which can be a barrier for some laboratories [98]. Despite these challenges, LC-MS/MS is mostly used for multiple mycotoxin detection in cereal grains and other complex matrices, and it does not require cleanup for sample preparation [99]. The LC-MS/MS technique was employed in this thesis as it could detect the presence of regulated and masked mycotoxins including other secondary metabolites in all grains analysed.

1.4.3 Control strategies for mould and mycotoxins in stored grains.

Monitoring changes in moisture content (MC) and temperature in stored grains is important, as they can significantly influence biological activities [100]. When the MC or a_w exceeds the safe threshold level of > 14 % or 0.70 a_w for most grains,

it can lead to dew formation, hydrolysis, respiration, and enzyme and microbial activities within the grain [101].

Temperature variations inside the silo can lead to moisture migration, where moisture moves from warmer to cooler areas. This can lead to concentrated pockets of high humidity, contributing to mould growth and spoilage. Elevated temperatures can accelerate respiration, leading to increased consumption of grain nutrients and a shorter shelf life [102,103]. Moisture and temperature sensors have been utilized to monitor mould spoilage in stored grains, but they have shown delayed responses [104]. The changing climate in the UK, characterized by rising temperatures and fluctuating seasonal rainfall, is anticipated to have a significant negative effect on cereal production and the food supply chain [105]. Therefore, it is crucial to implement appropriate rapid interventions.

Grain respiration maintains a constant CO₂ level during storage, but fungal development in the grain mass can cause a deviation from the steady state conditions. Therefore, CO₂ measurements have been used as a sensitive and early detector of mould spoilage initiation and pest activity [106]. Previous research at Cranfield University has explored various conditions for storing cereal grains to minimize spoilage fungi growth and mycotoxin production, using CO₂ or respiration measurement [107,108]. However, the models only considered two grains, wheat, and maize, with mycotoxigenic fungi strains of *Fusarium graminearum* and *Aspergillus flavus*, respectively, without taking into account the relationship between the initial mycobiota of grains, the real-time CO₂ production data during storage, and the final toxins produced in the grains. Other researchers have also attempted to use an integrated approach, including CO₂, relative humidity, and temperature measurements, to monitor grain deterioration [100,109,110]. However, the relationship between the real-time CO₂ measurements and the multiple mycotoxin contamination (including conjugated and emerging mycotoxins) in the cereal grains under different storage conditions has not been previously addressed in detail.

Modern management practices such as a Decision Support System (DSS) are necessary to prevent the development of fungal and mycotoxin hot spots in stored grains. A DSS is a sophisticated tool that integrates real-time data, predictive modelling, analytical techniques, and management recommendations to help farmers, agronomists, and policymakers make rapid and informed decisions to minimize mycotoxin contamination [111].

According to van Der Fels-Klerx et al. [111], the DSS is comprised of four different modules: (i) static information module (ii) scenario analysis module, (iii) dynamic module for forecasting mycotoxins, and (iv) dynamic module for real-time monitoring of moulds/mycotoxins in grain silos. This research focused on the fourth (iv) part of the DSS by using an integrated real-time monitoring approach in UK grains, focusing on ochratoxins and type A trichothecenes and their conjugated mycotoxins to generate real-time data for developing a DSS. The cost-benefit of different integrated monitoring approaches were analysed to ensure their suitability and sustainability for grain storage management.

1.5 Aim and Objectives

This PhD research aimed to generate the knowledge/data for the dynamic silo management module that would help to develop a complete integrated post-harvest Decision Support System (DSS) to improve the management of stored cereal grains by reducing fungal growth and mycotoxin contamination. This study proposes that by knowing the initial fungal population and mycotoxin content of grains at harvest and correlating this data with subsequent CO₂ production, moisture content, and temperature measurements during storage, we can improve the development of a decision support system (DSS) for stored grains.

The project has five interconnected objectives shown below.

Objective 1: To examine the fungal diversities of the initial mycobiota of temperate cereal grains (wheat/barley/maize/oat) grown in England and Northern Ireland.

Objective 2: To investigate the impact of storage conditions (temperature and water activity) on UK regulated, and conjugated mycotoxin concentrations in stored cereal grains.

Objective 3: To examine the relationship between CO₂ production at different optimum and marginal conditions (temperature and moisture content) for both fungal growth and mycotoxin production in stored cereals grains to identify low and high-risk storage conditions by using mini-silo and pilot scale testing with CO₂ sensors.

Objective 4: To examine if the CO₂ data from objective 3 can be combined with Cranfield's previous work on optimum and marginal boundary conditions for growth and toxin production to develop the decision support system.

Objective 5: To analyse the potential cost of such a DSS tool if it is economically affordable for grain farmers or companies to improve post-harvest management of cereals grains.

1.5.1 Novelty and linkage of project outputs

Several significant contributions have been made to the scientific community by achieving the objectives outlined for this research project. This thesis details these contributions through the presentation of eight chapters including three research articles. The interrelationships among them are shown in Figure 1-5 below.

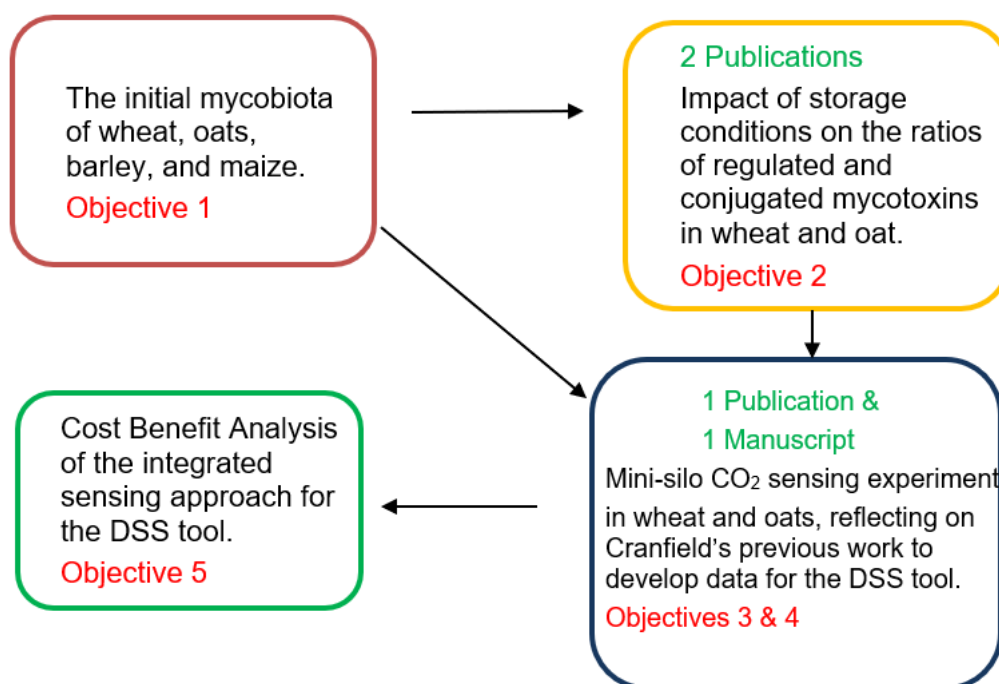


Figure 1-4: The Interconnections of the project outputs and articles/manuscripts presented in this thesis.

1.5.2 Outline of PhD Thesis

This doctoral thesis is structured in a paper-based format with eight chapters. Below is a brief description of the content of each chapter.

- Chapter 1 shows the background and motivation of the research project, the aims and objectives, and how the publications are linked with the aims and objectives.
- Chapter 2 describes the impact of different harvested years and regions on grains' initial mycobiota and mycotoxin profiles.
- Chapter 3 discusses the methodology and results of the impact of different environmental conditions on the ratios of regulated, conjugated, and emerging mycotoxins in stored oats. This chapter is published in the MDPI Toxin journal (doi.org/10.3390/toxins16040166).

Chapter 1

- Chapter 4 describes the methodology and results of the impact of different environmental conditions on the ratios of regulated, conjugated, and emerging mycotoxins in stored wheat. This chapter is published in the Mycotoxin Research journal (doi.org/10.1007/s12550-024-00541-6).
- Chapter 5 presents the methodology and results of the mini-silo sensing experiment where integrated CO₂ sensors were used to monitor real-time respiration production in stored naturally contaminated and *P. verrucosum* inoculated wheat to investigate if CO₂ produced can predict OTA contamination and identify the storage conditions with exceeded c limit. This chapter is published in the Food Chemistry journal (doi.org/10.1016/j.foodchem.2024.140590).
- Chapter 6 discusses the methodology and results of the mini-silo sensing experiment where integrated CO₂ sensors were used to monitor real-time respiration production in stored naturally contaminated and *F. langsethiae* inoculated oats to investigate if CO₂ produced indicates T-2/HT-2 toxin and OTA contamination and identify the storage conditions with exceeded legislative limits. This chapter is submitted for publication.
- Chapter 7 discusses the cost-benefit analysis of the integrated sensing DSS tool.
- Chapter 8 presents a general discussion of the research connecting all the chapters with the concluding remarks, contributions to existing knowledge, and recommendations for future research based on the findings detailed in this thesis.

Chapters 1 and 2 show the background information and initial mycobiota analyses of four grains (wheat, barley, oats and maize). Barley and wheat are classified as small cereal grains and are affected by similar *Fusarium* species, therefore, further experiments from chapters 3-6 focus only on wheat and oats. Results on maize experiments from objectives 2 and 3 were excluded from the thesis in order to meet the thesis submission deadline. However, these findings will be published.

Chapter 1

Experiment on how different storage conditions impact *Penicillium verrucosum* toxins in natural, irradiated and inoculated wheat from objective 2 was not included in the thesis to meet the thesis submission deadline. Also, the masked mycotoxin (ochratoxin alpha) was not available at the time of the experiment.

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2 The initial mycobiota and mycotoxin contamination of grains harvested in different years in the UK

2.1 Introduction

Yearly seasonal changes significantly impact the prevalence and types of fungal contamination. Cereal grains are susceptible to fungal attack during planting and harvest seasons due to high temperatures and humidity and are subsequently contaminated with mycotoxins [1,2]. Mycotoxins are toxic secondary metabolites produced by filamentous fungi that occur naturally, posing serious health risks to humans and animals and contributing to economic instability [3,4]. Geographic locations and their associated climatic conditions determine the seasonal fungal growth and mycotoxin production patterns. In the tropical and subtropical regions, characterised by warm and humid climates, there is a higher incidence of aflatoxin contamination. Conversely, temperate regions may experience higher occurrences of ochratoxin A and patulin, produced by *Penicillium* and *Aspergillus* species, during cooler and wetter seasons [5,6]. Pérez-Pizá et al. [7] reported that planting time influences the grain mycobiota and mycotoxin levels in Argentinian maize while Tralamazza et al. [8] studied how different regions/locations influence the fungal diversity in the mycobiota and mycotoxin contamination of harvested Brazilian wheat grains. They reported the prevalence of Deoxynivalenol from all regions while Zearalenone concentrations differed among regions.

Storage conditions during different seasons in a year influence fungal growth; improper storage during warm and humid periods can promote mycotoxin contamination. Therefore, the mycobiota of grains from the field before storage is important [2,9]. Several studies have shown how different interacting abiotic conditions in storage affect fungal growth and mycotoxin production in various cereal commodities [10–12].

The United Kingdom climate, monitored by the Met Office, has been trending towards warmer, wetter, and sunnier conditions in recent decades, with notable seasonal variations. These changes directly impact the development and

prevalence of microbial communities and mycotoxins in cereal crops [13]. During the colder or winter months (December–February) with a temperature range of 0–7 °C, lower temperatures and high humidity promote the growth of psychrotrophic and mesophilic fungi, such as *Penicillium* spp. and *Fusarium* spp which colonize grains in storage. The spring season (March–May), with increasing temperatures (5–15 °C) and moisture, can exacerbate these conditions, favouring fungal proliferation. The Summer months (June–August) with a temperature range of 12–25 °C is warmer and often wetter providing optimal conditions for the growth of thermophilic fungi- *Aspergillus* species. As temperatures drop (7–18 °C) and humidity levels remain high in September–November (Autumn months), the mycobiota of grains undergoes another transition. *Fusarium* species, prevalent during cooler and moist conditions, become more dominant producing a range of mycotoxins, including trichothecenes, zearalenone, and fumonisins [13]. Therefore, post-harvest grains should be dried to < 15 % MC and water activity of < 0.70 a_w with adequate storage environment monitoring for long-term storage [14]

Future changes in temperature, precipitation, and atmospheric CO₂ concentrations are expected to increase the risk of mycotoxin contamination in cereal crops. These changes may also affect the geographical distribution of specific cereals, mycotoxigenic fungi, and their mycotoxins [15,16].

Table 2-1 shows the seasonal comparison between England and Northern Ireland while Table 2-2 shows the values of the mean air temperature and total precipitation for England and Northern Ireland from 2019–2021.

Chapter 2

Table 2-1: Seasonal comparison between England and Northern Ireland.

	ENGLAND			NORTHERN IRELAND		
	Temperature	Rainfall	Sunshine	Temperature	Rainfall	Sunshine
Spring (March-May)	9–15 °C	light	Increasing sunshine hours	7–13 °C	Moderate	Slightly fewer sunny days
Summer (June - August)	18–25 °C	Dry + occasional rain showers	Long daylight hours	15–20 °C	Frequent rain	Fewer sunny days
Autumn (September - November)	10–17 °C	Increased rainfall	Decreasing sunshine hours	8–14 °C	Persistent rainfall	Cloudier with shorter daylight hours
Winter (December - February)	2–8 °C	Generally wet	Short days with limited sunshine	3–7 °C	High rainfall	Very limited sunshine

source: Met Office, [13].

Table 2-2: Seasonal mean air temperature and total precipitation in England and Northern Ireland from 2019-2021.

Year/seasons		ENGLAND				NORTHERN IRELAND			
		Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter
2019	T (°C)	9.2	16.3	10.1	5.6	8.3	14.4	8.9	5.9
	R (mm)	166	262	361	184	294	342	329	245
2020	T (°C)	9.7	16.0	10.9	5.9	8.54	13.9	9.7	5.1
	R (mm)	89.0	272	256	341	125	397	360	412
2021	T (°C)	7.7	16.2	11.6	4.2	7.5	15.0	10.9	4.1
	R (mm)	175	178	229	341	209	193	285	372

Source: Met Office National Climate Information Centre. Met Office, [17]. T- Temperature. R- Rainfall.

The knowledge of the climatic conditions and identification of the critical conditions within storage facilities is crucial for developing a decision support system tool to minimise mould growth and mycotoxin contamination in cereal grains. Sadyś et al. [18] reported the impact of climate change on selected fungal spores' distribution in the West Midlands of the UK during two years of contrasting weather. Fungal spores can be described as 'wet' or 'dry' according to the type of weather associated with their occurrence in the air. They found that wet fungal spores such as *Didymella*, *Leptosphaeria* and *Rusts* species were more sensitive to weather changes, with statistically significant differences observed between the two years of study, compared to "dry" fungal spores (*Alternaria*, *Cladosporium* and *Epicocum* species). However, how different years of harvest with different climatic conditions affect the fungal community in UK cereal grains is not well documented. Therefore, in this chapter, we investigated how the diversity of mycotoxigenic fungi in cereal grains varies across three harvested years (2019, 2020, 2021) with different climatic conditions in two regions (England and Northern Ireland) of the UK.

2.2 Methodology

2.2.1 Grains

Wheat, barley, maize, and oat grains were collected from different farms in Bedfordshire (England) and Northern Ireland farm outlets harvested in the 2019, 2020, and 2021 seasons (Table 2-3). Grains were stored 1-3 months post-harvest before collection for the experiment. 250 g of grains were sampled from 25 kg bulked grains into clean, sealable bags. The initial moisture content and water activity of the grains were analysed, and all grains were stored at 4 °C until further analysis.

Table 2-3: Grains collected from different regions and harvested years in the United Kingdom

Country	Grains code	Location	Harvested Year	Variety
	WHEAT ENG '19	ENGLAND	2019	Zulu
	WHEAT ENG '20	ENGLAND	2020	Belepi
	WHEAT ENG '21	ENGLAND	2021	Zulu
	WHEAT NI '20 1	NORTHERN IRELAND	2020	-
	WHEAT NI '20 2	NORTHERN IRELAND	2020	-
	BARLEY ENG '19	ENGLAND	2019	Propino
	BARLEY ENG '20 1	ENGLAND	2020	Planet
	BARLEY ENG '20 2	ENGLAND	2020	Malt
	BARLEY ENG '21 1	ENGLAND	2021	Craft
	BARLEY ENG '21 2	ENGLAND	2021	Planet
	BARLEY NI '20	NORTHERN IRELAND	2020	-
	MAIZE ENG '19	ENGLAND	2019	-
	MAIZE NI '20	NORTHERN IRELAND	2020	-
	OAT ENG '19	ENGLAND	2019	Mascani
	OAT ENG '21 1 OAT ENG '21 2	ENGLAND	2021	Mascani
	OAT NI '21 1 OAT NI '21 2	NORTHERN IRELAND	2021	-

2.2.2 Moisture content (MC) analysis

10 g of sub sampled grains were weighed into glass Petri dishes, dried in the oven overnight at 105 °C, and cooled in a desiccator containing silica gel. The final weights of the petri dishes with the samples were obtained. The percentage MC (dry weight) was calculated on a wet weight basis as shown in Equation 2-1 and the graph plotted in Figure 2-1. Air oven method AAOE Official method 925.09 [19] was used for the moisture content analysis of the grains.

$$\% \text{ Moisture Content (MC)} = \frac{(\text{Tin,Lid + Sample}) \text{ initial weight} - (\text{Tin,Lid + Sample}) \text{ final weight}}{\text{Sample weight (Tin,lid+ sample - Tin + Lid)}} \times 100 \quad (2-1)$$

2.2.3 Water activity (a_w) analysis

The grains were analysed for a_w using a water activity meter (4 TE Decagon devices, Aqualab Inc., Pullman, WA, USA). The meter was calibrated after the instrument warmed up for 15 minutes. Calibrated standards were placed in the sample chamber, and readings were allowed to stabilise at 25°C. Measurements were taken thrice for each standard with values in the range of ± 0.003 to verify accuracy.

The cell was half-filled with whole grains and placed in the meter's sample chamber. The a_w readings were taken in triplicate from the same sample from the same batch at 25 °C and the graph was plotted as shown in Figure 2-2.

2.2.4 Fungal enumeration and isolation

2.2.4.1 Media preparation

Malt Extract Agar (MEA, Oxoid CM59)

MEA agar is a general growth media suitable for the cultivation, isolation and maintenance of microorganisms. 50 g agar (Oxoid, Basingstoke, Hampshire, UK) + 5% chloramphenicol (an antibiotic that reduces bacteria growth- Thermo Fischer Scientific, Geel, Belgium) were mixed with 1000 mL distilled water in a Duran bottle.

Dichloran Glycerol 18 (DG18 Oxoid CM0729)

DG18 agar is a selective medium used to isolate and count microorganisms. It has low water activity and is used for identifying xerophilic moulds. 31.5 g agar (Oxoid, Basingstoke, Hampshire, UK) + 5% chloramphenicol was mixed with 1000 mL distilled water in a Duran bottle.

Coconut cream agar medium (CCA)

This medium was used for screening strains for their ability to produce mycotoxins under UV (365 nm) light. A 50% creamed coconut homogenate (KTC Edibles, Wednesbury, UK) was dissolved in 100 mL distilled water + 2% Technical agar + 5% chloramphenicol were mixed in a Duran bottle.

Yeast extract sucrose agar (YES)

This medium was used as a conductive medium for the production of mycotoxins by mycotoxigenic fungi. 60 g of yeast extract sucrose (Oxoid, Basingstoke, Hampshire, UK), + 8g Technical agar (SIGMA-ALDRICH, Dorset, UK) were mixed in 400 mL distilled water.

Peptone agar

Peptone agar media is a nutrient-rich medium used to grow microorganisms.

15 g peptone agar (SIGMA-ALDRICH, Dorset, UK) was mixed in 1000 mL distilled water. 9 mL was dispensed into 25 mL universal glass bottles.

Tween 80 solution

Tween 80 can enhance the growth and production of microbial extracellular metabolites in fungi. 2 drops of Tween 80 solution (SIGMA-ALDRICH, Dorset, UK) were added to 500mL of distilled water in a Duran bottle.

All media were autoclaved at 121 °C for 15 minutes. The molten media were aseptically poured into 9cm Petri dishes in a laminar hood and allowed to cool while the peptone and tween 80 water were allowed to cool.

2.2.4.2 Serial dilution method

1 g of each whole grain was soaked in 9 mL sterile peptone water in a 25 mL universal bottle for 3 hours. One mL of this bottle was serially diluted (by ten-fold dilution) to the 0.0001M solution bottle. Sterile tips were used between each dilution and the universal bottles were shaken with vortex. 100 µL of the dilution aliquots were reversely plated onto DG18+ and MEA+ media in 3 replicates and spread out with a sterile metal spreader. The plates were incubated at 25 °C for 7 days. Fungi genera were identified visually with a stereoscope (Olympus 308136, Wild M7A, Heerbrugg, Switzerland) and morphological characteristics [20]. Colonies were counted with a colony counter (Gallenkamp, Cambridge UK) and expressed as $\log_{10}$ CFUs/g-dry weight.

2.2.4.3 Direct plating method

25 grains were directly inoculated on both media. Another 25 grains were surface sterilized with sodium hypochlorite (NaOCl- 0.4% v/v) for 2 minutes, rinsed, and dried with sterile filter paper in both cases. 5 grains were inoculated at equidistant from each other on each plate of 5 replicates to allow the growth of internal fungi

colonisers of the grains. Plates were incubated at 25 °C for 7 days. Fungal genera were identified visually with stereoscope and morphological characteristics and were calculated for frequency of isolation using Equation 2-2.

$$\% \text{ Frequency of isolation} = \frac{\text{number of contaminated grains}}{\text{Total number of contaminated grains}} \times 100 \quad (2-2)$$

2.2.4.4 Fungal colonies identification

Fungal genera were identified using a stereoscope by observing macroscopic morphological characteristics such as colony elevation (raised, flat, or umbonate), texture (velvety, powdery, cottony), colour (both surface and reverse), and overall shape or growth pattern (circular, spreading, zonate). The stereoscope also allows for the visualisation of reproductive structures like conidiophores or sporangia, whose arrangement, shape, and pigmentation aid in identification. These features, when compared to taxonomic keys or reference images in Samson et al. [20], enable preliminary genus-level identification

2.2.5 Fungi isolation from cereals.

2.2.5.1 Fungi isolation on Coconut Cream Agar (CCA)

Spores and mycelia of fungi from mixed fungal colonies on 7-day-old plates of DG18+ and MEA+ media were inoculated onto newly prepared MEA+ plates and incubated at 25 °C for 7 days to obtain a pure culture of potentially mycotoxigenic strains for mycotoxin analysis. Spores of *Penicillium* and *Aspergillus* isolates (pure culture) were suspended in 5 mL of sterile tween water and vortexed. 5 µL was inoculated centrally onto the CCA media. *Fusarium* mycelia were taken with a sterile plug and centrally inoculated onto YES media. All inoculated plates were incubated at 25 °C for 7-10 days. After 10 days, CCA plates were viewed under a handheld UV light lamp of 365nm longwave UV light (EA-160/FBE, Spectroline, USA) for fluorescence to confirm if the fungal strains were mycotoxigenic.

2.2.5.2 Mycotoxin extraction using LC-MS/MS

Three - four plugs of the *Fusarium* strains on the seven-day-old YES media were transferred into weighed sterile 2 mL Eppendorf tubes. The strains were mixed with extraction solvent-acetonitrile: water: formic acid (79:20:1 v/v/v) and incubated for 90 minutes at 300 rpm at 25 °C on a rotary shaker (Mini Shaker VMR, Leighton Buzzard, UK). It was centrifuged at 2500 rpm for 10 minutes. 500 µL of supernatant was transferred into Liquid Chromatography tandem mass spectrometry (LC-MS/MS) vials and was diluted with acetonitrile: water: formic acid (20:79:1 v/v/v) and vortexed. 5 µL was injected into the LC-MS/MS for metabolomic analysis.

The LC-MS/MS machine used in this study was a LQTRAP 5500+ MS/MS system (SCIEX) equipped with a Turbo V electrospray ionization (ESI) source, coupled to an ExionLC AD System (SCIEX, Framingham, MA, USA). Chromatographic separation was performed at 27 °C on a Gemini C18-column, 100 × 4.6 mm (Phenomenex, Torrance, CA, USA). The chromatographic method, as well as chromatographic and mass spectrometric parameters, were adapted from Malachová et al. [21]. The mass spectrometry parameters used are outlined in Table 2-4 below.

Table 2-4: Optimised MS/MS parameters for the analysed mycotoxins, including precursor ions, product ions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP).

Components	Precursor Ion (<i>m/z</i>)	Product Ion (<i>m/z</i>)	Retention Time	DP (V)	CE (V)	CXP (V)
Deoxynivalenol	355.1	59.2	1.95	70	40	13
	355.1	265.2	1.95	70	22	10
DON-3-Glc	517.3	427.1	1.85	80	30	11
	517.3	59.1	1.85	80	25	7
T-2 toxin	484.3	215.2	6.79	57	29	17
	484.3	185.1	6.79	27	33	11
HT-2 toxin	447.4	345.1	6.10	131	27	20
	442.2	323.2	6.10	50	15	16
Zearalenone	317.1	175.0	7.15	110	34	13
	317.1	131.2	7.15	110	42	8
Nivalenol	371.1	59.1	0.95	75	42	7
	371.1	281.1	0.95	75	22	15

DON-3-Glc: Deoxynivalenol-3-glucoside

2.2.6 Statistical analysis

Data were analysed with JMP® Pro 16 software. They were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene tests. When data failed the normality test, they were transformed logarithmically to achieve normality for the moisture content and a_w data. Transformed data were normally distributed; therefore, a one-way ANOVA was used to find differences between groups. A Tukey HSD test was used to analyse the significant differences in the mean values for each grain sample. For the non-normal data, a nonparametric comparison for each pair using the Wilcoxon method showed differences among fungal populations and toxin for each sample.

2.3 Results

2.3.1 Moisture content (MC) and water activity (a_w) analyses

The moisture contents (w/w %) of all grains were below 14 % except for the WHEAT ENG '21 autumn harvest with 16.1 % MC, BARLEY ENG '19 (14.9 %) and MAIZE ENG '19 (14.5 %). The mean values of the MC of all grains are shown in Figure 2-1.

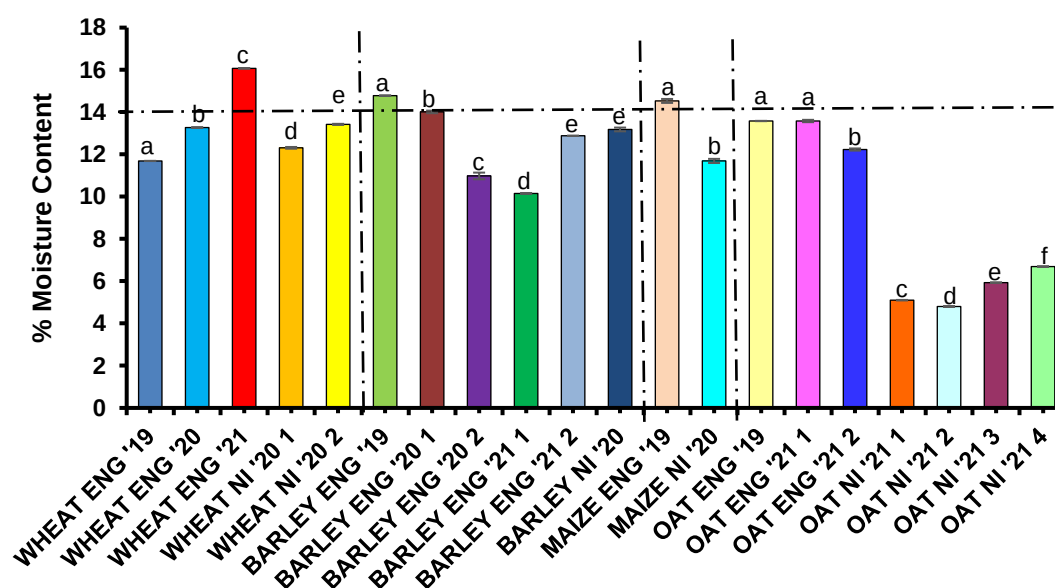


Figure 2-1: Mean values of moisture content of cereal grains with standard error bars (n=3). Different letters showed significant differences in values for each grain type for mean comparison using the Tukey-Kramer HSD test. The dotted horizontal line represents the safe threshold risk moisture content level (15 %) in most cereal grains. Vertical dotted lines separate individual grains.

The water activities of most grains were below the safe threshold risk level of 0.70 a_w recommended for safe storage conditions without the initiation or growth of spoilage fungi and mycotoxin production except Wheat ENG '21, Wheat NI '20 2, Barley ENG '19, Barley ENG '20 1, Barley ENG '21 2, Maize ENG '19, Oat ENG

'19 and Oat ENG '21 seasons. The mean values of the a_w of all grains are shown in Figure 2-2.

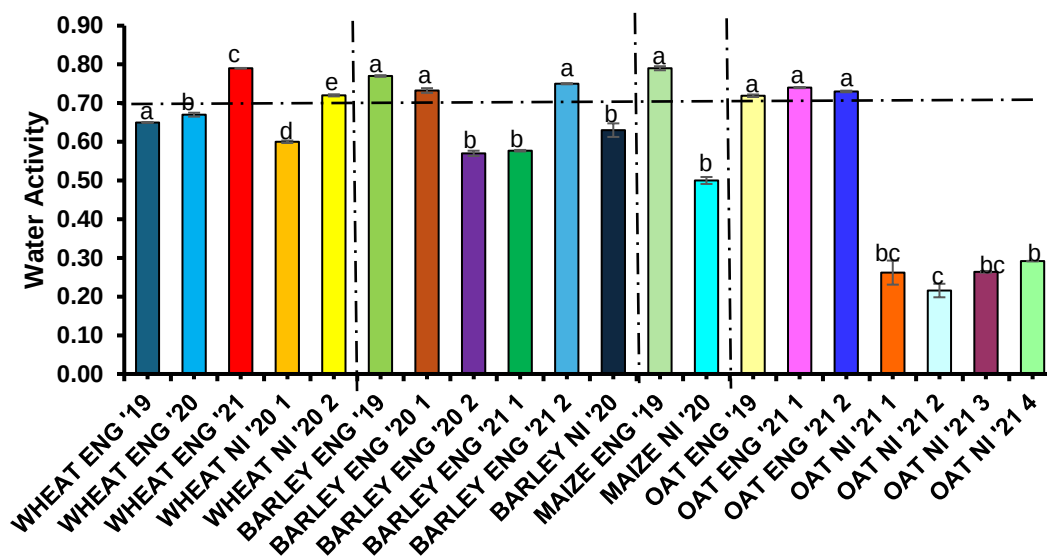


Figure 2-2: Mean values of water activity of cereal grains with standard error bars ($n=3$). Different letters showed significant differences in values for each grain type for mean comparison using the Tukey-Kramer HSD test. The dotted horizontal line represents the safe threshold risk water activity level ($0.70 a_w$) in grains. Vertical dotted lines separate individual grains. a_w values are in temperature range of 24 - 25 °C.

2.3.2 Initial mycobiota of the grains

2.3.2.1 Fungal populations

Table 2-4 below shows the populations of the potentially mycotoxigenic fungal genera in all grain samples.

Penicillium and *Fusarium* populations were highest in WHEAT ENG 2019. However, no significant differences in the *Penicillium* populations among the three harvested years and between the two regions in year 2020.

BARLEY ENG 2021 also had the highest *Penicillium* populations, with no significant differences in the *Penicillium* populations among the three harvested years and between the two regions. *Fusarium* was only found in BARLEY ENG 2020 year while *A. sect. Nigri* was only present in BARLEY NI 2020.

No potentially mycotoxigenic fungal populations as found in the oat grains from England in 2019 and 2021, however, *Penicillium* and *Fusarium* populations were present in OAT NI 2021.

Only *Penicillium* populations were found in MAIZE ENG 2019. There was no mycotoxigenic fungal growth on the Maize NI 2020 as it was dominated by the *Aureobasidium* fungal genus.

Table 2-5: Mean values ($n=3 \pm \text{SD}$) of the initial fungal populations ($\log_{10}$ CFUs/g) in the grains.

Grains	<i>Penicillium</i>		<i>Fusarium</i>		<i>A. section Nigri</i>	
	MEA+	DG18+	MEA+	DG18+	MEA+	DG18+
WHEAT ENG '19	3.7 ± 1.8	3.6 ± 1.9	ng	2.9 ± 1.7	ng	ng
WHEAT ENG '20	3.3 ± 1.9	3.4 ± 1.8	ng	ng	ng	ng
WHEAT ENG '21	2.6 ± 1.6	2.6 ± 1.6	ng	ng	ng	ng
WHEAT NI '20	2.8 ± 1.7	2.8 ± 1.4	ng	ng	ng	ng
BARLEY ENG '19	2.9 ± 0.0	3.2 ± 0.0	ng	ng	ng	ng
BARLEY ENG '20	2.6 ± 0.7	2.3 ± 2.2	3.6 ± 3.5	ng	ng	ng
BARLEY ENG '21	5.6 ± 5.6	5.6 ± 5.6	ng	ng	ng	ng
BARLEY NI '20	3.1 ± 0.0	3.1 ± 0.0	ng	ng	3.1 ± 0.0	ng
OAT ENG '19	ng	ng	ng	ng	ng	ng
OAT ENG '21	ng	ng	ng	ng	ng	ng
OAT NI '21	5.3 ± 5.2	5.5 ± 5.5	ng	2.3 ± 2.0	ng	ng
MAIZE ENG '19	2.6 ± 2.5	2.6 ± 2.5	ng	ng	ng	ng
MAIZE NI '20	ng	ng	ng	ng	ng	ng

SD: Standard deviation. MEA+: Malt Extract Agar media. DG18+: Dichloran Glycerol Agar media. +: chloramphenicol. ENG: England, NI- Northern Ireland. '19, '20 and '21- years 2019, 2020 and 2021. ng: no growth.

2.3.2.2 Fungal isolations

Several fungal genera were found in the grains. Each contaminated media plate shows the co-occurrence of different fungal species in each grain's natural mycobiota. An important observation is that despite the fungal growth, some of the grains retained their germinative trait (Figure 2-3).

Generally, the fungal diversities and populations isolated from the four grains were predominantly *Penicillium*, *Alternaria*, *Aspergillus* section *Nigri*, *Cladosporium*, *Aspergillus* section *Aspergillus* (formerly called *Eurotium*), and *Mucor* species. The relative abundance (%) of each fungus is shown in Figures 2-4, to 2-7 below.

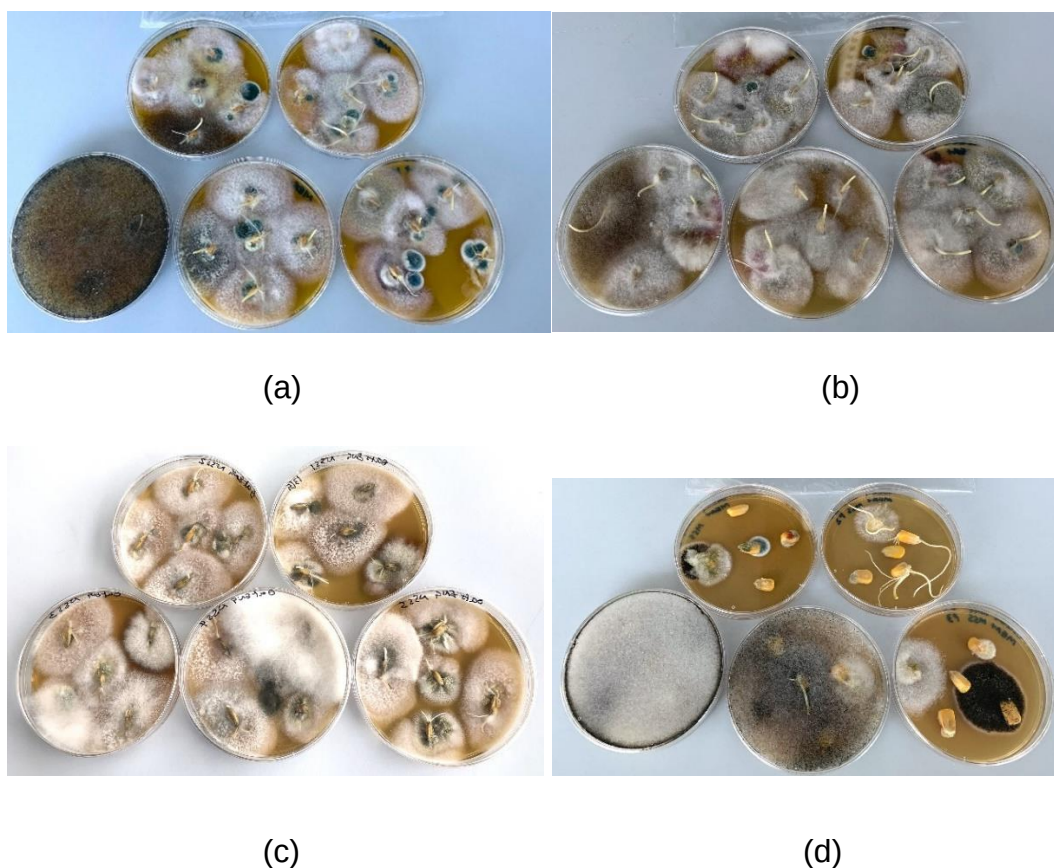


Figure 2-3: Pictorial representations of the initial microbiota in (a) wheat grains (b) barley grains (c) oat grains (d) maize grains for non-surface sterilised treatment on Malt Extract Agar (MEA + chloramphenicol) using the direct plating method after seven days of incubation at 25 °C.

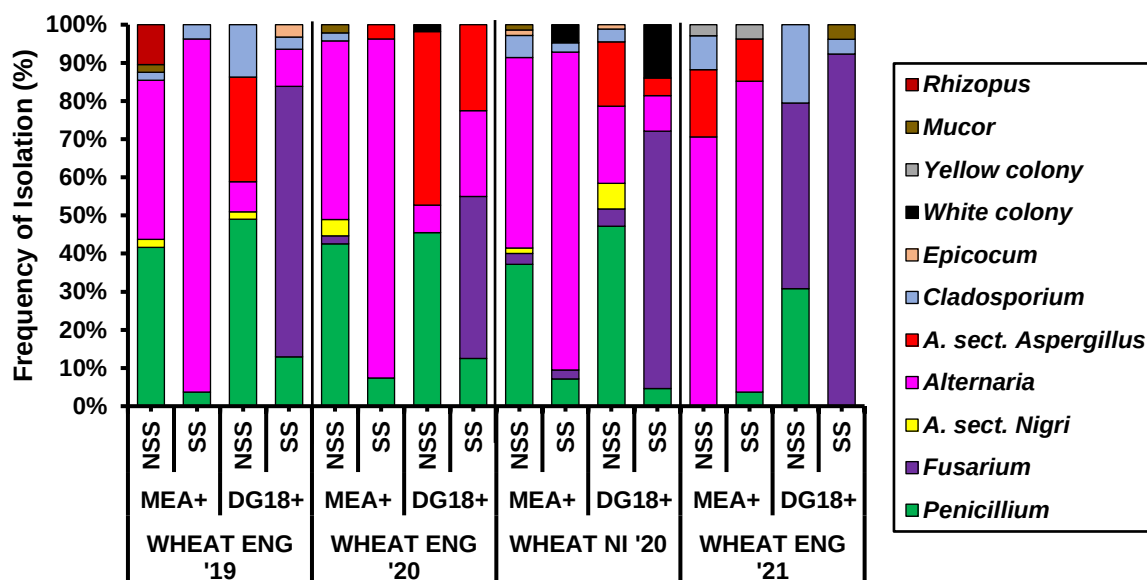


Figure 2-4: Frequency of isolation (%) of fungal diversity from naturally contaminated wheat grains on MEA+ and DG18+ media with surface sterilisation (SS) using 4% sodium hypochlorite (NaOCl) and non-surface sterilisation (NSS). ENG: England, NI- Northern Ireland, '19, '20 and '21- Year 2019, Year 2020 and 2021. MEA+: Malt Extract Agar+ chloramphenicol, DG18+: Dichloran-Glycerol 18+ chloramphenicol.

Figure 2-4 shows that the composition of the initial fungal community was relatively similar in all wheat samples. *Alternaria* was mostly isolated in all wheat samples and higher in the surface sterilised (SS) treatments on MEA+ media. It was highest (96%) in the WHEAT ENG '19 than in other England years of 2020 (88%) and 2021 (81%).

Besides, WHEAT NI '20 was contaminated with more fungal genera for both surface sterilisation (SS) and non-surface sterilization (NSS) treatments on the two media compared to WHEAT ENG '20.

A. sect. Aspergillus was highest (40%) in WHEAT ENG '20 for the NSS on DG18+ media compared to other England years and the WHEAT NI '20 grains.

Cladosporium and *Mucor* were present in all wheat samples but at a relatively low level. *Epicocum* was relatively low and present in WHEAT NI '20 but absent in WHEAT ENG '20. It was also isolated from the WHEAT ENG '19 sample.

Among the mycotoxigenic fungal genera, *Penicillium* species had the highest frequency of isolation in all wheat samples except WHEAT ENG '21 (Figure 2-4)

Fusarium has the highest isolation in the surface sterilised (SS) treatments compared to the non-surface sterilisation (NSS) treatments. It was the highest (96%) in the WHEAT ENG '21. Among all the England years, WHEAT ENG '21 has no *A. sect. Nigri*, while WHEAT NI '20 had the highest frequency of isolation of *A. sect. Nigri* 7% on DG18+ media compared to WHEAT ENG '20 with 4% level of isolation on MEA+ media. *A. sect. Nigri* was not an internal coloniser of wheat as it was only found on the non-surface sterilised treatment in all wheat grains.

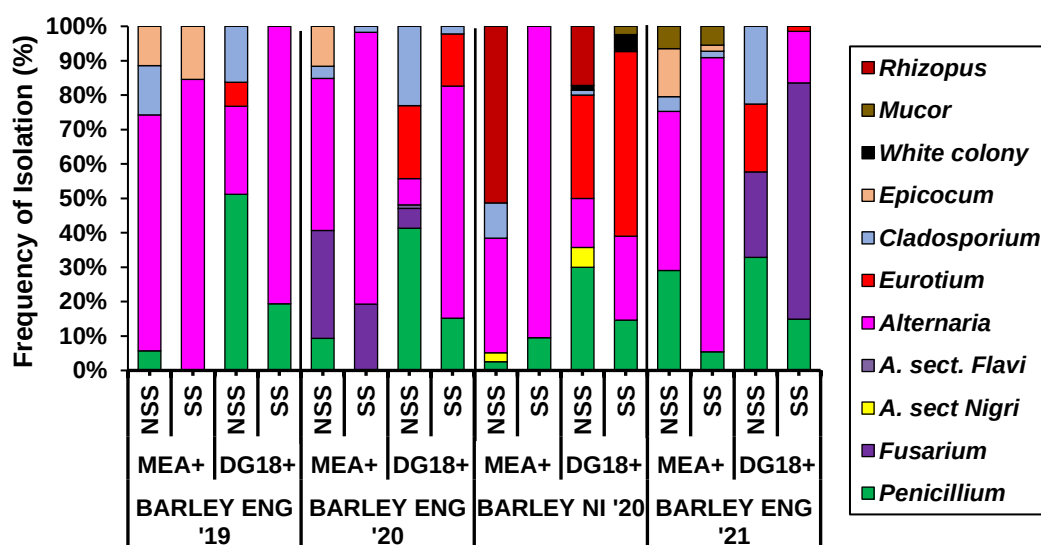


Figure 2-5: Frequency of isolation (%) of fungal diversity from naturally contaminated barley grains on MEA+ and DG18+ media with surface sterilisation (SS) using 4% sodium monochloride (NaOCl) and non-surface sterilisation (NSS). ENG: England, NI- Northern Ireland, '20 and '21- years 2020 and 2021. MEA+: Malt Extract Agar+ chloramphenicol, DG18+: Dichloran-Glycerol 18 +

chloramphenicol. BARLEY ENG '19 has the least fungal genera contamination among England barley grains. *Alternaria* was predominant and highest in the surface sterilised barley grains on MEA+ media. *A. sect. Aspergillus (Eurotium)* was mostly found on DG18+ media and was relatively high in BARLEY NI '20. Also, *Rhizopus* was only found in BARLEY NI '20. It however had no *Cladosporium* growth, unlike the England season barley grains.

Penicillium genera were found in all barley samples without co-occurrence with other fungal genera in BARLEY ENG '19. *Fusarium* genera were found in BARLEY ENG '20 and BARLEY ENG '21.

BARLEY ENG '20 had *A. sect. Flavi* and *Fusarium* which were both absent in BARLEY NI '20. However, *A. sect. Nigri* was isolated from BARLEY NI '20 (Figure 2-5).

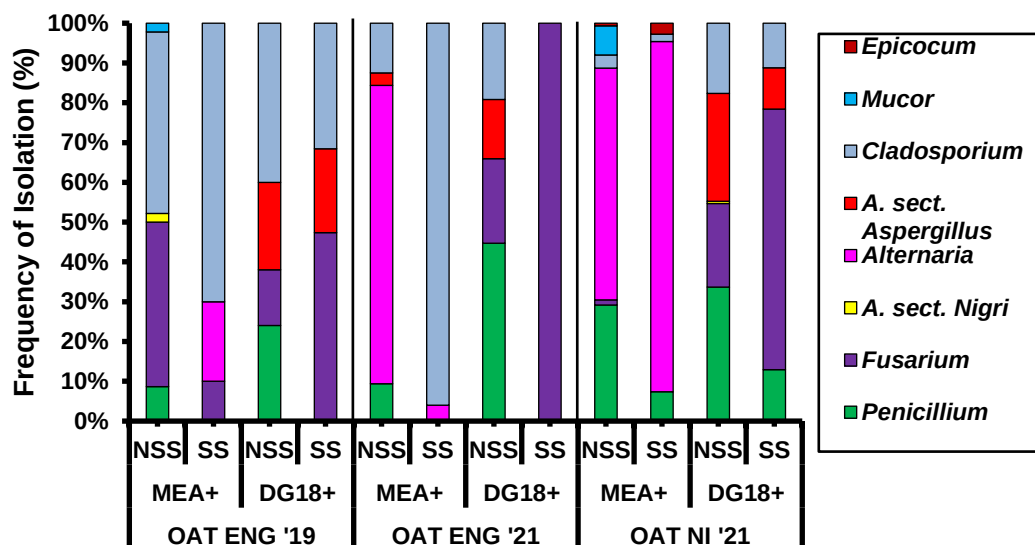


Figure 2-6: Frequency of isolation (%) of fungal diversity from naturally contaminated oat grains on MEA+ and DG18+ media with surface sterilisation (SS) using 4% sodium monochloride (NaOCl) and non-surface sterilisation (NSS). ENG: England, NI- Northern Ireland, '20 and '21- years 2020 and 2021. MEA+: Malt Extract Agar+ chloramphenicol, DG18+: Dichloran-Glycerol 18 + chloramphenicol.

Figure 2-6 shows that among the spoilage fungal genera, *Cladosporium* mostly dominated OAT ENG '19 than OAT ENG '21 and isolation was low in OAT NI '21. *Alternaria* was mostly found on MEA+ media and was highest in OAT NI '21. *Mucor* was isolated from the NSS treatment on MEA+ in both OAT ENG '19 and OAT NI '21 while *Epicocum* was only isolated from OAT ENG '21.

Fusarium species mostly dominated oats in the surface sterilised treatment for both media and it was more isolated in OAT ENG '19 than in OAT ENG '21 oats.

A. sect. Nigri was relatively low and found in OAT ENG '19 (4 %) and in OAT ENG '21 (1 %).

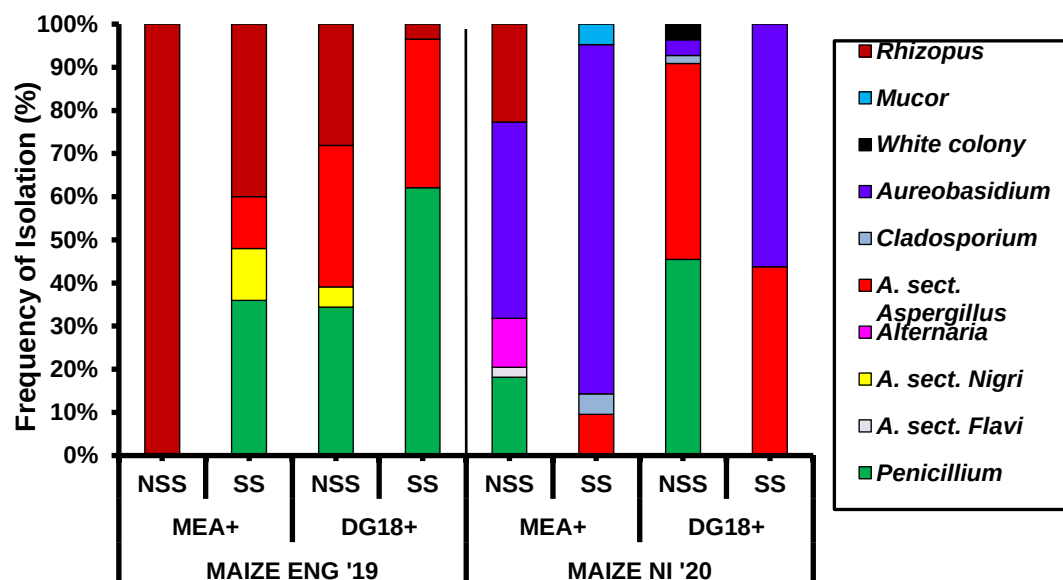


Figure 2-7: Frequency of isolation (%) of fungal diversity from naturally contaminated maize grains on MEA+ and DG18+ media with surface sterilisation (SS) using 4% sodium monochloride (NaOCl) and non-surface sterilisation (NSS). ENG: England, NI- Northern Ireland, '20 and '21- years 2020 and 2021. MEA+: Malt Extract Agar+ chloramphenicol, DG18+: Dichloran-Glycerol 18 + chloramphenicol.

Rhizopus was relatively abundant in MAIZE ENG '19, while *Aureobasidium* dominated MAIZE NI '20. *Alternaria* and *Cladosporium* were low and were isolated only in MAIZE NI '20 while *A. sect. Aspergillus* (*Eurotium*) was highest on the DG18+ media for both treatments.

Penicillium was mostly isolated from both maize seasons. *A. sect. Nigri* was isolated in MAIZE ENG '19 while *A. sect. Flavi* was isolated in MAIZE NI '20. No mycotoxigenic fungi were reported for MAIZE ENG '19 for the NSS treatment on MEA+ media as the plate was overcrowded with *Rhizopus*, as shown in Figure 2-10 above. It was interesting that no internal coloniser was observed in MAIZE NI '20 as no fungal growth on the surface sterilized treatment for both media (Figure 2-7).

2.3.3 Preliminary screening for mycotoxigenicity using Ultraviolet light

The fluoresced plate (a) in Figure 2-19 below represents the mycotoxigenic *Penicillium* (control) strain while plates (b) and (c) are the isolated *Penicillium* strains from wheat grains without fluorescence around the fungal colonies meaning that the isolated *Penicillium* strains are not positive for mycotoxin production.

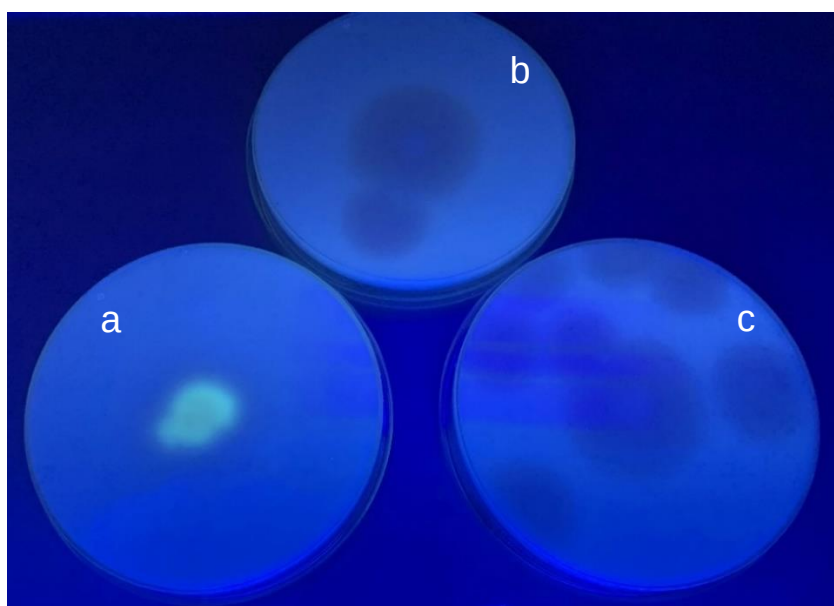


Figure 2-7: *Penicillium* species isolated from wheat grain under Ultraviolet (UV) light. Plate a represents the mycotoxigenic *Penicillium* (control) strain that fluoresce under UV light. Plates b and c are the isolated *Penicillium* strains from wheat grains without fluorescence

2.3.4 Mycotoxins in grains

Table 2-6 below shows the limit of detection and limit of quantification of *Fusarium* toxins analysed.

Chapter 2

Table 2-6: The apparent recovery and limit of detection and quantification of the mycotoxins.

	Deoxynivalenol	DON-3-glucoside	Nivalenol	HT-2 toxin	T-2 toxin	Zearalenone
Apparent recovery (%)	80	84	60	100	98	100
LOD(ngg ⁻¹)	4.0	4.0	0.8	1.5	0.7	0.2
LOQ(ngg ⁻¹)	13.0	13.0	2.6	5.0	2.4	0.6

LOD- limit of detection. LOQ- limit of quantification.

In Table 2-7, WHEAT ENG '19 is contaminated with a *Fusarium* strain having the highest DON and NIV levels while *Fusarium* strains isolated from other grains had DON levels below the limit of detection. None of the *Penicillium* and *Aspergillus* strains isolated from all grains fluoresced under the UV light.

Table 2-7: Mycotoxin production and UV fluorescence by fungal strains isolated from grains

Fungal strain code/type	Grain source code	Method/Treatment/media	Fluorescence on CCA media	Mycotoxins (ngg ⁻¹) by LC-MS/MS	
				DON	NIV
24P- <i>Fusarium</i>	BARLEY ENG '20 2	DP/NSS/DG18+	NA	< LOQ	4.2 ± 3.8
4AP- <i>Fusarium</i>	WHEAT ENG '19	DP/NSS/MEA+	NA	25.8 ± 11.8	10 ± 5.9
25W- <i>Fusarium</i>	BARLEY ENG '20 2	SD/DG18+	NA	< LOQ	ND
14P- <i>Fusarium</i>	MAIZE ENG '19	SD/DG18+	NA	< LOQ	ND
2GGa- <i>Penicillium</i>	BARLEY ENG '19	DP/NSS/DG18+	-	NA	NA
24G- <i>Penicillium</i>	BARLEY ENG '20 2	DP/NSS/DG18+	-	NA	NA
20A- <i>A. sect. Flavi</i>	BARLEY NI '20	DP/NSS/DG18+	-	NA	NA
2WA- <i>A. sect. Nigri</i>	BARLEY ENG '20 1	DP/NSS/DG18+	-	NA	NA
4GGa1	WHEAT ENG '19	DP/NSS/MEA+	-	NA	NA

Chapter 2

<i>Penicillium</i>						
4GGa2	WHEAT ENG '20	DP/NSS/MEA+	-	NA	NA	
A. sect. Nigri						
13Gb	MAIZE ENG '19	DP/SS/DG18+	-	NA	NA	
A. sect. Nigri						
17G	BARLEY ENG '20 1	DP/NSS/DG18+	-	NA	NA	
Penicillium						
7G	MAIZE NI '20	SD/DG18+	-	NA	NA	
Penicillium						

DP- Direct plating. SD- Serial dilution. SS- surface sterilised. NSS- non-surface sterilised. CCA- Coconut cream agar media. DON- Deoxynivalenol. NIV- Nivalenol. MEA- Malt Extract Agar. DG18- Dichloran Glycerol 18. +: Chloramphenicol. ND- Not detected. NA- Not applicable. Mean values of mycotoxins $\pm$ Standard deviation (n=3).

Table 2-8 below shows that wheat grains from England were more contaminated with DON, DON-3-glucoside, and Nivalenol than wheat from Northern Ireland. WHEAT ENG '20 had DON-3-glucoside levels greater than its precursor DON among all wheat analysed.

Nivalenol was mostly found in the barley grains with the highest concentrations in BARLEY NI '20. MAIZE NI '20 has no measurable toxin contamination (below LOD) compared to MAIZE ENG '19.

OAT ENG '19 is more contaminated with DON, T-2 toxin, and HT-2 toxin than Oats from Northern Ireland.

Table 2-8: Mean values of the initial mycotoxin profile of grains

Grains	Mycotoxins (ng g ⁻¹)					
	Deoxynivalenol	DON-3-glucoside	Nivalenol	HT-2 toxin	T-2 toxin	Zearalenone
WHEAT ENG '19	18.3 ± 1.7 ^a	16.3 ± 0.8 ^a	47.0 ± 30.8 ^a	6.6 ± 4.7	< LOD	< LOD
WHEAT ENG '20	18.8 ± 4.5 ^a	27.8 ± 2.8 ^a	64.3 ± 54.7 ^a	< LOD	< LOD	< LOD
WHEAT NI '20 1	46.8 ± 39.9 ^a	24.1 ± 20.5 ^a	48.0 ± 39.4 ^a	< LOD	< LOD	< LOD
WHEAT NI '20 2	45.5 ± 35.3 ^a	41.8 ± 38.9 ^a	< LOD ^b	< LOD	< LOD	5.5 ± 3.4
BARLEY ENG '19	< LOQ	< LOQ	30.5 ± 22.7 ^a	7.2 ± 1.1	< LOD	< LOD
BARLEY ENG '20 1	< LOD	< LOD	70.8 ± 60.2 ^a	6.6 ± 4.6	< LOQ	< LOD
BARLEY ENG '20 2	20.3 ± 16.6 ^a	< LOQ	34.0 ± 2.8 ^b	< LOD	< LOD	0.8 ± 0.6
BARLEY NI '20	< LOQ	17.5 ± 16.5 ^a	116.6 ± 69.6 ^b	< LOQ	5.0 ± 3.2	< LOD
MAIZE ENG '19	107.1 ± 45.3 ^a	25.3 ± 16.7 ^a	62.2 ± 10.7	< LOD	< LOD	5.2 ± 2.7
MAIZE NI '20	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
OAT ENG '19	26.9 ± 4.0 ^a	< LOD	< LOD	92.2 ± 11.0 ^a	61.2 ± 6.8	< LOD
OAT ENG '21	< LOQ	< LOQ	< LOQ	52.7 ± 5.1 ^a	49.1 ± 3.9	< LOD
OAT NI '21 1	< LOQ	< LOD	< LOD	< LOD	8.8 ± 0.2	< LOD
OAT NI '21 2	< LOD	< LOD	< LOD	30.1 ± 21.8 ^b	< LOD	< LOD
OAT NI '21 3	< LOQ	< LOD	< LOD	25.4 ± 10.5 ^b	< LOD	< LOD
OAT NI '21 4	< LOQ	< LOD	< LOD	19.2 ± 11.5 ^b	< LOD	< LOD

LOD- limit of detection. LOQ- limit of quantification. Using a non-parametric comparison for each pair (Wilcoxon method), the same letter shows no significant differences in mycotoxin concentrations among each grain of the same group (within the column).

2.4 Discussion

Fungal contamination in cereal grains is influenced by various factors, including yearly seasonal variations and geographical locations [22]. Warmer and more humid conditions, typical of summer and autumn, often promote fungal proliferation. Conversely, colder and drier conditions in winter and spring can inhibit/minimise fungal growth [23]. This study highlights the fungal diversities in wheat, barley, oats, and maize grains from three harvested years and two regions (England and Northern Ireland) in the United Kingdom.

The moisture content of grains after harvest plays a major role in the safety of the grains in storage. Three grains from England had their MC above 14 % while eight grains from England had a_w above the safe threshold. Only one grain (WHEAT NI '20) from Northern Ireland exceeded 0.70 a_w Figure 2-2. This suggests that these grains are susceptible to fungal spoilage during long-term storage. The meteorological data from Table 2-2 shows that NI had wetter autumns and winters than England in 2020 and 2021. The MC and a_w indicate that grains from NI were efficiently dried after harvest. The mean values of the MC and a_w for all grains are in Table B-1 in Appendix B.

Generally, the initial fungal communities in wheat, barley, and oats have similar fungal genera such as *Alternaria*, *Penicillium*, *Fusarium* and *Aspergillus*. However, the maize's initial mycobiota is slightly different. The surface sterilisation of grains was done to minimise the microbial load of grains allowing the identification of internal colonisers or mycoflora of grains. *Alternaria* genera was relatively in abundance in both surface and non-surface sterilised wheat and barley grains for all years and regions on the MEA+ media (**Error! Reference source not found.** and **Error! Reference source not found.**). *Fusarium* genera were the dominant internal coloniser of the wheat and oat grains as shown in the surface-sterilized grains on the DG18+ media (**Error! Reference source not found.** and **Error! Reference source not found.**). It was interesting to note that *A. sect. Nigri* was the only internal colonizer found in maize grain from England. *Penicillium* was isolated from all grains irrespective of years and regions and it had the highest populations among the mycotoxigenic fungal genera. The fungal

populations result showed no significant differences in the populations of the fungal genera among each grain irrespective of the year or location. The mean values of the potential mycotoxigenic fungal populations and isolations in all grains are in Table **B-2** and Table **B-3** in Appendix B.

A similar study reported the order of the dominance of fungal genera in wheat from Tunisia cities as *Alternaria*, *A. sect. Aspergillus (Eurotium)* and *Aspergillus* [24]. However, the order of the dominant fungal genera in wheat from our study are *Alternaria*, *Penicillium*, and *Fusarium*. This was similar to the findings of Belkacem-Hanfi et al. [25], in durum wheat from Northern Tunisia. This implies that *Alternaria* (also known as field fungi) remain a strong competitor as the dominant fungi in wheat irrespective of the geographic or climatic conditions.

Similar to Jedidi et al.'s [24] study, we reported a diverse fungal community in maize, different from wheat and barley. Due to its higher moisture content at harvest, maize was more contaminated with *Rhizopus* than other cereals. MAIZE ENG '19 had the highest presence of *Rhizopus*; this was a reflection of its water activity value of 0.79 a_w , as compared to MAIZE NI '20, which had 0.50 a_w .

Aureobasidium species are characterized by their yeast-like growth and smooth, darkly pigmented colonies that can vary from white to pink or black due to melanin production [26]. They often thrive in moist conditions and at a wide range of temperatures (5–35 °C). Certain strains are used as biocontrol agents against plant pathogens, leveraging their competitive ability to inhibit harmful microorganisms on crops [27]. These reasons may be related to its dominance in the MAIZE NI '20 (**Error! Reference source not found.**) and the lack of other fungal genera in the fungal populations observed through the serial dilution analysis.

Ultraviolet light (UV) can be used as a preliminary test to detect the potential presence of certain mycotoxins; however, it cannot quantify their toxicity level [28]. The UV light was used to screen the isolated fungi from the grains to examine if they were mycotoxigenic. Our findings showed that the isolated *Penicillium* and *Aspergillus* strains from the grains did not fluoresce under the UV light when matched with their respective toxigenic strains as shown in Figure **2-7**.

Fusarium toxins were analysed using the LC-MS/MS as shown in Table 2-7. The initial mycotoxin profile for most regulated toxins is shown in Table 2-8. DON-3-Glc concentrations in WHEAT ENG '20 were higher than the concentrations of its precursor toxin. This result aligns with the findings of Kostelanska et al. [29] who reported higher DON-3-Glc than DON in beer and brewing intermediate products from barley. Findings from studies with other cereals opposed our findings reporting low levels of DON-3-G compared to DON concentrations [30–32]. Barley grains from Northern Ireland in 2020 had the highest nivalenol contamination compared to their England counterparts and surprisingly, were contaminated with DON-3-G without its precursor toxin. Despite being an acetylated form of T-2 toxin, HT-2 dominated all the oats samples with Oats from England 2019 (OAT ENG '19) having the highest contamination. Some studies also supported our findings reporting higher concentrations of HT-2 toxin than T-2 toxin in oat grains [33,34]. Maize from Northern Ireland, year 2020 had less contamination compared to Maize from England in 2019. This was reflected in the fungal population and fungal strain isolation results as shown in Table 2-7 respectively.

This chapter discussed the fungal diversities and mycotoxin contamination found in the four cereal grains after harvest. It emphasizes the importance of efficient drying prior to storage to minimize or prevent fungal growth that can develop from the field and continue in storage.

2.5 Conclusions

Yearly seasonal variations play a pivotal role in influencing fungal growth and mycotoxin production. Temperature, humidity, moisture levels, geographic and climatic factors determine the types and levels of mycotoxin contamination. Comprehensive knowledge of these seasonal effects is vital for devising effective strategies to minimise mycotoxin risks and ensure food safety. Although below maximum legislative limits, this chapter suggested the potential risk of *Fusarium* toxins contamination in wheat, barley, and oats from England and Northern Ireland for long-term storage. Other conclusions include;

- 15% and 45% of the grains analysed had moisture content and a_w respectively exceeding the safe threshold risk level for grain storage.
- Spoilage fungal genera were relatively in abundance in all grains compared to the mycotoxigenic fungal strains.
- The frequency of the isolation of *Penicillium* genera was the highest among the mycotoxigenic fungal strains.
- The predominant *Aspergillus* species found in most grains was *A. section Nigri*.
- There were no significant differences in the *Penicillium* populations among years and regions for wheat and barley grains.
- Non-surface sterilised grains were contaminated with more fungal genera than the surface sterilised grains suggesting contamination from the field and during harvest.
- Despite the high frequency of isolation, the *Penicillium* strains isolated from the grains were not mycotoxigenic. Two *Fusarium* strains each from wheat and barley had measurable values for Deoxynivalenol and Nivalenol.
- The initial mycotoxin profile shows that the wheat grains were mostly contaminated with DON, its glucoside (Deoxynivanol-3-glucoside), and Nivalenol among the regulated mycotoxins.
- Maize from Northern Ireland, year 2020 was less contaminated than Maize from England year 2019.
- The oat grains analysed were mostly contaminated with HT-2 toxin.

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3 Impact of environmental conditions on the concentrations of trichothecenes, their glucosides, and emerging *Fusarium* toxins in naturally contaminated, irradiated, and *Fusarium langsethiae*- inoculated oats

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Abstract:

Trichothecenes produced by *Fusarium* species are commonly detected in oats. However, the ratios of the concentrations of free trichothecenes and their conjugates and how they are impacted by different interacting environmental conditions are not well documented. This study aims to examine the effect of water activity (0.95 and 0.98 a_w) and temperature (20 and 25 °C) stress on the production of T-2 and HT-2 toxins, deoxynivalenol and their conjugates, as well as diacetoxyscirpenol (DAS). Multiple mycotoxins were detected using Liquid chromatography-tandem mass spectrometry from 64 contaminated oat samples. The highest concentrations of HT-2-glucoside (HT-2-Glc) were observed at 0.98 a_w and 20 °C and were higher than other type A trichothecenes in the natural oats' treatments. However, no statistical differences were found between the mean concentrations of HT-2-Glc and HT-2 toxins in all storage conditions analysed. DAS concentrations were generally low and highest at 0.95 a_w and 20 °C, while deoxynivalenol-3-glucoside levels were highest at 0.98 a_w and 20 °C in the naturally contaminated oats. Emerging mycotoxins such as beauvericin, moniliformin, and enniatins mostly increased with a rise in water activity and temperature in the naturally contaminated oats treatment. This study reinforces the importance of storage a_w and temperature conditions in the high risk of free and modified toxin contamination of small cereal grains.

Keywords: T-2 toxin; HT-2 toxin; diacetoxyscirpenol; deoxynivalenol; masked mycotoxins; LC-MS/MS; oat grains.

Key Contribution: This article investigated the concentrations of T-2 toxin; HT-2 toxin; deoxynivalenol; their glucosides; and diacetoxyscirpenol for the first time under different storage conditions to determine the relative risk of their contamination in stored oats.

3.1 Introduction

Fusarium langsethiae, originally referred to as a 'powdery *Fusarium poae*', is a major producer of the type A trichothecenes T-2 toxin (T-2) and HT-2 toxin (HT-2), initially discovered in Norwegian cereals [1,2]. It is characterized by spores morphologically similar to *Fusarium poae*, with a similar mycotoxin profile to *Fusarium sporotrichioides* [3]. The occurrence of *Fusarium langsethiae* and its mycotoxins in a wide range of cereals and cereal products such as oats, wheat, and barley has been extensively examined [4–9].

Diacetoxyscirpenol (DAS), mainly produced by *Fusarium langsethiae* and mostly found in cereal grains, has been assessed for its toxicity to humans and animals by the European Food Safety Authority (EFSA) [10]. DAS, also called anguidine, belongs to group A of trichothecenes, having a similar structure and toxicity to T-2 toxin, causing lymphoid necrosis, haematotoxicity, and gastrointestinal toxicity [11,12]. Considered a severe threat to human, animal, and plant health, DAS is enlisted for research in the 'Select Agents and Toxins' list of the Centres for Disease Control and Prevention, United States [13]. DAS has been found to reduce feed intake and body weight with other cytotoxic effects such as oral lesions in broiler breeders fed with a DAS-contaminated diet [14]. Its occurrence in cereal and cereal-based foods is not well documented.

Deoxynivalenol (DON) has been found in oats and was reported to be mainly produced by *F. graminearum*, which is the main causative agent of *Fusarium* head blight (FHB) in small cereal grains, especially in temperate regions [6,15,16]. Unlike *F. langsethiae*, it is a strong pathogen that can penetrate plant cell walls, causing aggressive colonisation [17,18]. DON has been reported to co-occur with its glucoside, DON-3-G, in cereal-based products and with acetylated forms in oats [19–21]. However, these studies did not investigate the impact of storage conditions on the concentrations of these mycotoxins in oats.

As a major contaminant of oats, T-2 toxin can be rapidly metabolized to HT-2 toxin [22]. The effects of both toxins include a weakened immune system, growth retardation in exposed animals, and cytotoxicity in the human small intestine, which are well-detailed in the JECFA report on HT-2 and T-2 toxins and other

related studies [23–25]. Due to their toxicity, indicative levels for the sum of T-2 and HT-2 ($\mu\text{g/kg}$) have been set by the European Commission at 1000 $\mu\text{g/kg}$ in unprocessed oats (with husks), with a tolerable daily (TDI) intake of 0.02 $\mu\text{g/kg}$ of body weight per day [26].

T-2 and HT-2 can be metabolized to their modified forms, such as HT-2-glucoside, T-2-glucoside, 3'-hydroxy-T-2, T-2 tetraol, and 3'-hydroxy-T-2 triol [23,27]. While these metabolites are less toxic, the glucosides can be converted back to their native form in the mammalian gastrointestinal tract to regain their toxicity [27]. These modified forms are not readily detected by conventional analytical techniques, making it difficult to predict the total mycotoxin levels in harvested grains [28]. Grains that seem to be clean or healthy may be contaminated with high levels of mycotoxins and their modified forms due to the frequent occurrence of *Fusarium* species in small-sized cereal grains without visible traces of infection [29,30]. It is therefore important to understand *Fusarium* infection either pre- and/or post-harvest and examine how environmental factors influence mycotoxin contamination of grains by *Fusarium langsethiae*.

A storage temperature of 25 °C and 0.995 a_w was reported as the optimum temperature for the growth of two strains of *Fusarium langsethiae* on oat-based media [31]. The highest concentrations of T-2 and HT-2 in oat grains *in situ* were reported at 25 °C in the wettest condition of 0.97 a_w by Mylona and Magan, [32]. However, while these studies showed the growth of *Fusarium langsethiae* and the production of T-2 and HT-2 in oats *in vitro* and *in situ*, they have not shown how their concentrations vary alongside their conjugates in stored oats under different environmental conditions. Therefore, as maximum legislative levels for T-2 and HT-2 toxins would be set in the year 2024 by the European Union Commission, it is imperative to examine if different environmental conditions influence free T2, HT-2, and their conjugated forms in stored oat grains.

The objectives of this study were to examine the impact of storage conditions, namely water activities of 0.95 a_w and 0.98 a_w and temperatures of 20 °C and 25 °C, on (a) the concentrations of free T-2 toxin and HT-2 toxin, DON, and their respective glucosides and (b) the concentrations of DAS and other emerging

mycotoxins in both naturally contaminated and irradiated oat grains inoculated with *Fusarium langsethiae* to ascertain any potential increases in toxicity in the oat grains.

3.2 Materials and Methods

3.2.1 Fungal isolates

Fusarium langsethiae (2390/2391), known to produce T-2 and HT-2 toxins, was used in this study. The strain was maintained in glycerol/water (70:30, v/v) at –20 °C in the culture collection of the Magan Centre of Applied Mycology, Cranfield University. *Fusarium langsethiae* was grown on malt extract agar (MEA) + chloramphenicol (anti-bacterial agent) incubated at 25 °C for 7 days and then sub-cultured on V8 agar at 25 °C for 7 days for active sporulation.

3.2.2 Oat grains and moisture adsorption curve analysis

Oat grains (with husk) were collected from Bedfordshire farms (2019 harvest). Grains were stored at 4 °C ten months before the experiment, where 5 kg of grains were exposed to 12.5–15 kGys (STERIS, Bradford, UK) to reduce microbial contamination from the grains while retaining their germination capacity. The initial mycobiota of the natural and irradiated oats were analysed by placing five grains equidistant on five MEA+ media on 9 cm Petri plates in a sterile flow bench incubated at 25 °C for 7 days. The fungal identification was visually evaluated with a stereoscope.

In total, 10.0 g of both treated oat grains were placed in 25 mL universal glass bottles and a known amount of water (0.1–3.5 mL) was added to the grains. The bottles were tightly sealed to reduce moisture loss and stored at 4 °C for 24 h with regular shaking. The samples were equilibrated at room temperature for 1 hour and analysed for water activity with a water activity meter (4 TE Decagon devices, Inc AQUALAB, Pullman, WA, USA) and moisture content, with the samples oven-dried at 105 °C overnight. Three replicates were analysed for each

grain treatment. The amount of added water was plotted against a_w values to modify the grains to the targeted a_w levels (see Figure 3-1). The relationship between the moisture content (MC) (dry weight basis) and a_w values was also plotted and noted as discussed by Chulze et al. [38].

3.2.3 Grain inoculation

Spores were harvested aseptically from a 7-day-old *F. langsethiae* plate scraped with a sterile spatula with sterile Tween 80 water (0.05% v/v). Then, 10 mL of the suspension was transferred into a sterile tube and shaken to obtain a homogenous mix. The spores' concentrations were counted and calculated with a Thoma cell counting chamber. The spore suspension was further diluted with Tween 80 water to achieve 10^6 spores/mL targeted concentrations. Targeted a_w levels of 0.95 and 0.98 a_w for the natural and irradiated oat grains were calculated using the moisture adsorption curve. A total of 120 g of grains was mixed with sterile water (1 mL less for spore inoculum) and stored at 4 °C for 24 h to equilibrate. The control treatments were also modified with sterile water. The equilibrated grains were dispensed (15 g) into 40 mL clear glass volatile organic analysis (VOA) vials with sealable polytetrafluoroethylene (PTFE) caps containing a silicone septum for gas exchange. Grains with the same a_w levels were placed in 12 L poly-propylene environmental chambers with 2 × 500 mL beakers of glycerol–water solution to maintain the target equilibrium relative humidity (ERH) of the atmosphere for each a_w level. The chambers were stored at temperatures of 20 and 25 °C for 17 days. The grains were dried at 55 °C overnight, ground, and stored at –20 °C before further analysis.

3.2.4 Mycotoxin analysis

3.2.4.1 Chemical reagents

HT-2-toxin-3-O- β -D-glucoside was synthesized by Michlmayr et al. [51]. Other mycotoxin standards supplied by the Institute for Global Food Security, Queen's University Belfast (QUB), UK were purchased from Romer Labs (Tulln, Austria). They include DON-3-G, HT-2 toxin, T-2 toxin, DON, 15-acetyl-deoxynivalenol (15-AcDON), 3-acetyl-deoxynivalenol (3-AcDON), DAS, enniatin A (ENN A), enniatin A1 (ENN A1), enniatin B (ENN B), enniatin B1 (ENN B1), moniliformin (MON) beauvericin (BEA), and nivalenol (NIV). LC-MS/MS-grade methanol, acetonitrile, and formic acid (Honeywell, Seelze, Germany); ammonium acetate (MS grade, Sigma-Aldrich, Darmstadt, Germany); and glacial acetic acid (Sigma-Aldrich, Burlington, MA, USA) were used. Water was purified successively by reverse osmosis and using a Milli-Q plus system from Millipore (Merck, Molsheim, France).

3.2.4.2 Sample preparation and extraction

The initial mycotoxin concentrations of the oat grains were analysed. Following the storage experiment, 64 contaminated oat samples (including replicates) were analysed for mycotoxins. A volume of 4 mL of extraction solvent (acetonitrile (ACN): water (H₂O): acetic acid (HAc) 79: 20:1, v/v/v) was added to 1.00 g of ground oat grains. Extraction was carried out for 90 min using a multitube vortex (VWR DVX-2500, VMR International Ltd Leicestershire, UK), followed by centrifugation for 15 minutes at 5000 rpm on a Rotina 380R centrifuge (Hettich, Tuttlingen, Germany). Then, 200 μ L of the extract was diluted with 800 μ L of dilution solvent (ACN/H₂O/acetic acid, 20:79:1, v/v/v). The diluted extracts were then filtered into LC-MS/MS vials using a 0.22 μ m PTFE filter, with 5 μ L of the diluted extract injected into the LC-MS/MS system for analysis.

3.2.4.3 LC-MS/MS parameters

The LC-MS/MS machine used in this study was a LQTRAP 5500+ MS/MS system (SCIEX) equipped with a Turbo V electrospray ionization (ESI) source, coupled to an ExionLC AD System (SCIEX, Framingham, MA, USA). Chromatographic separation was performed at 27 °C on a Gemini C18-column, 100 × 4.6 mm (Phenomenex, Torrance, CA, USA). The chromatographic method, as well as chromatographic and mass spectrometric parameters, were adapted from Malachová et al. [52]. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was performed in the time-scheduled MRM mode both in positive and negative polarities in one chromatographic run per sample by scanning two fragmentation reactions per analyte. Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM of ammonium acetate and were composed of water/methanol/acetic acid at 89:10:1 (v/v/v; eluent A) and 2:97:1 (v/v/v; eluent B), respectively, with a sample injection volume set at 5 µL, with a total runtime of 7.5 min. The gradient elution program for the elution of mycotoxins was as follows: 0 min 5% B, 0.5 min 5% B, 2.5 min 70% B, 3.5 min 95% B, 5 min 95% B, 7.5 min 5% B. The mass spectrometry parameters used are outlined in Table 3-1 below.

Chapter 3

Table 3-1: Optimised MS/MS parameters for the analysed mycotoxins, including precursor ions, product ions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP).

Mycotoxins	Precursor ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)
Regulated mycotoxins					
Deoxynivalenol	297.1	249.1	91	21	20
	297.1	203.2	91	21	20
T-2 toxin	484.3	215.2	76	29	18
	484.3	185.1	76	31	11
HT-2 toxin	442.3	263.1	71	19	14
	442.3	215.1	71	19	22
Masked Mycotoxins					
3-Acetyldeoxynivalenol	397.3	59.2	-60	-38	-8
	397.3	307.1	-60	-20	-7
15-Acetyldeoxynivalenol	339.1	321.3	81	13	18
	339.1	261.1	81	17	14
Deoxynivalenol-3-Glucoside	517.2	427.1	-115	-30	-11
	517.2	457.2	-115	-20	-19
HT-2-Glc	604.3	323.1	101	17	16
	604.3	263.1	101	23	14
Emerging mycotoxins					
Beauvericin	801.3	784.3	141	27	14
	801.3	244.1	141	43	12
Diacetoxyscirpenol	384.2	307.3	86	17	16
	384.2	247.3	86	21	14
Enniatin A	699.4	682.4	10	27	24
	699.4	210.2	10	39	22
Enniatin B	657.3	640.3	10	27	22
	657.3	196.1	10	39	10
Enniatin B ₁	671.3	654.4	6	27	22
	671.3	196.1	6	41	22
Enniatin A ₁	685.4	668.5	11	27	12
	685.4	210.1	11	39	10
Moniliformin	96.9	41.2	-5	-38	-14
Nivalenol	371.1	281.1	-90	-20	-15
	371.1	59.1	-90	-50	-7

m/z: mass-to-charge ratio.

3.2.4.4 Optimised LC-MS/MS method validation

The optimised LC-MS/MS method for the analysis of mycotoxins in oats was validated based on the acceptable performance criteria of analytical methods set and updated by European Commission regulation No. 2021/808/EC [32]. The performance characteristics evaluated were linearity (r^2), limit of detection (LOD), limit of quantification (LOQ), matrix effect or the signal suppression/enhancement (SSE), recovery of the extraction process (RE), absolute recovery (R_A), and repeatability. Extraction and apparent recoveries were determined from five different lots of oats spiked in quintuplicate on three days, with the multi-mycotoxin working standard solution.

A working standard of 0.5 µg/mL (for DON-3-G and the enniatins) and at 1 µg/mL (for other toxins listed in Section 5.4.1) was prepared from the individual intermediate standards, diluted with ACN/H₂O (50:50, v/v) to achieve the desired concentration of each analyte in the working standard. Quantification was performed via external calibration using an eight-point calibration curve achieved by serial dilutions of the multi-analyte working standard solution. Data were further processed using Analyst® 1.7.1 and SCIEX OS-Q 3.0. The validation procedure was adapted from Siri-Anusornsak et al. [53].

The apparent recovery (R_A), matrix effect (SSE), and extraction efficiency (RE) were calculated using the Equations below.

$$\text{SSE (\%)} = \frac{\text{area (sample spiked after extraction)}}{\text{area (neat solvent standard)}} \times 100 \quad (3-1)$$

$$\text{RA (\%)} = \frac{\text{area (sample spiked before extraction)}}{\text{area (neat solvent standard)}} \times 100 \quad (3-2)$$

$$\text{RE (\%)} = \frac{\text{RA}}{\text{SSE}} \times 100 \quad (3-3)$$

3.2.5 Statistical Analyses

JMP® Pro 16, and Statistica 14.0.1 software were used for data analysis. Data were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene tests. When data failed the normality test, data were transformed to achieve normality. Transformed data were normally distributed; therefore, a one-way ANOVA was used to find differences between groups. Also, nonparametric comparisons for each pair using the Wilcoxon method showed differences among each toxin pair for each treatment. A Tukey HSD test was used to analyse the significant impact of the interactions of the storage conditions and treatments on the DAS and HT-2-glucoside concentration ratios. The linearity assumption and the normal distribution of residuals were examined, resulting in normal plots of the residuals. Concentrations of analytes <LOD and <LOQ are assigned with values of LOD/2 and LOQ/2, respectively, for the calculation of the mean concentration and statistical analysis [35]. Statistical analyses performed were considered significant when p values were < 0.05.

3.3 Results

3.3.1 Moisture adsorption curve (MAC) for natural and irradiated oats.

The MAC graph for both oat treatments is similar as shown below.

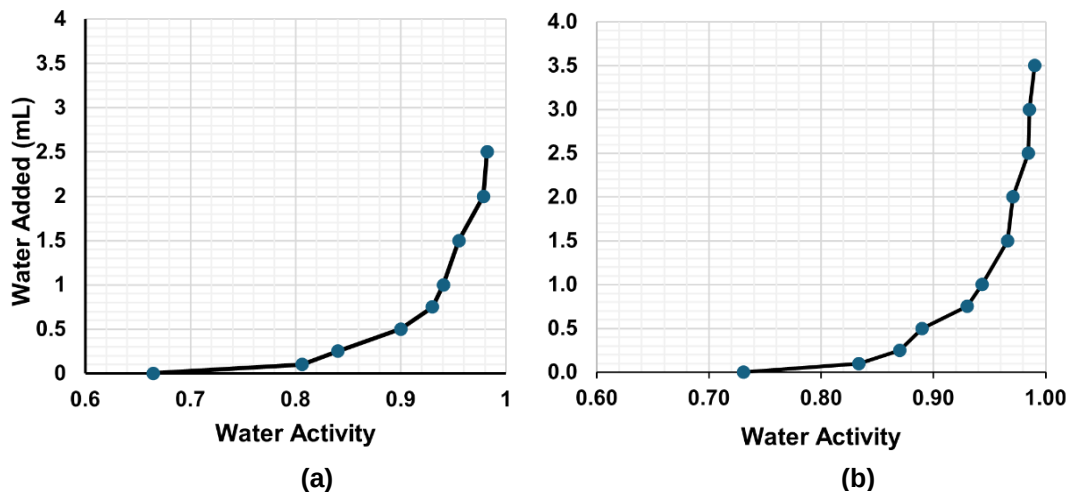


Figure 3-1: Moisture adsorption curves for 10g of (a) natural oats (d) irradiated oats grains.

The MAC graph is used to calculate the equivalent amount of water from their respective a_w level which is then modified for grain rehydration depending on the weight of the grain.

3.3.2 Fungal growth in stored oats



(a)

(b)

Figure 3-2: Pictorial representation of fungal growth in stored oat grains at 20 °C (a) *Penicillium* at 0.95 a_w and (b) *Fusarium* at 0.98 a_w .

Figures 3-2 a and b show *Penicillium* and *Fusarium* growth in the naturally contaminated oats after 18 days of storage respectively. The irradiated grains had no fungal growth on MEA+ media (Figure C-1 in Appendix C).

3.3.3 Method validation performance in the oat matrix

The multi-analyte extraction methodology was based on a ‘dilute-and-shoot’ approach. This is a sample preparation technique where samples are diluted and directly injected into an instrument for analysis. The extraction efficiency (R_E) of each analyte was within the acceptable range (70–120%), according to the amended guideline set by European Commission regulation No. 2021/808/EC [33] and showed good relative standard deviation (RSD) values of <15%. The goodness of fit of the calibration curve for each analyte was acceptable, with r^2 values (coefficient of determination) of > 0.990. The relative standard deviation of the within-laboratory reproducibility (RSD_{WLR}), the matrix effect or the signal suppression/enhancement (SSE), and the apparent recovery (R_A %) were calculated from the average of 75 replicates of the five different lots

of oats spiked in quintuplicate across three separate days. The trueness and precision represented by the relative standard deviation (RSD%) of the method were satisfactory (<14%) for the validated analytes, except for nivalenol (NIV). The limit of detection (LOD) and limit of quantitation (LOQ) for the analytes ranged from 1 to 19 µg/kg and 3 to 63 µg/kg, respectively, which were lower than the minimum acceptable levels for the regulated mycotoxins in unprocessed oats [34]. The mean values of the R_A , R_E , RSD, LOD, and LOQ for each analyte are shown in Table 3-2. The relative expanded measurement uncertainties for each analyte are shown in Table C-1 in Appendix C.

Table 3-2: Mean values (n = 75) of the inter-day precision within-lab reproducibility (WLR) method validation for wheat grains.

Components	RSD (%)	R_A (%)	SSE (%)	R_E (%)	LOD (ng/g)	LOQ (ng/g)
Moniliformin	6	87	116	74	2.0	6.7
15-Acetyldeoxynivalenol	13	57	65	95	2.8	9.2
3-Acetyldeoxynivalenol	3	95	94	99	2.2	7.3
Beauvericin	5	94	92	101	2.6	8.6
Diacetoxyscirpenol	5	93	89	100	1.9	6.4
Deoxynivalenol	3	96	96	101	3.7	12.4
DON-3-Glucoside	5	69	88	77	1.7	5.5
Enniatin A	7	86	84	103	1.0	3.4
Enniatin A1	5	89	91	98	1.6	5.4
Enniatin B	9	97	101	98	5.1	17.0
Enniatin B1	7	94	96	98	2.8	9.2
HT-2 toxin	3	94	96	98	4.1	13.6
HT-2-Glc	5	93	93	95	5.6	15.8
Nivalenol	22	80	85	92	19.0	63.2
T-2-toxin	2	94	94	99	3.4	11.4

R_A —apparent recovery, R_E —extraction efficiency, RSD—relative standard deviation, SSE—signal suppression/enhancement, LOD—limit of detection, LOQ—limit of quantitation. n = average values of 75 replicates from the 5 different lots of oats.

3.3.4 Initial mycotoxin concentrations of oats

The initial average concentrations of DON, T-2, HT-2, and HT-2 glucoside in the naturally contaminated oats before the storage experiment were 26.9 ± 3.9 ng/g, 61.2 ± 6.8 ng/g, 92.2 ± 10.9 ng/g, and 32.8 ng/g, respectively. One-way ANOVA showed significant differences between the concentrations of HT-2 and HT-2-Glc ($p < 0.05$).

3.3.5 Impact of a_w and temperature on the concentrations of T-2 toxin, HT-2 toxin, and HT-2-Glc in all oat treatments

The concentrations of HT-2-Glc were 1.5 times higher than the concentrations of HT-2 toxin at 0.95 a_w at 20 °C in the naturally contaminated oats (control samples). However, their concentrations had no significant differences ($p > 0.05$). T-2 toxin, HT-2 toxin, and HT-2-Glc concentrations were below the limit of detection (LOD) or limit of quantitation (LOQ) at 25 °C for both water activity levels examined.

Despite *F. langsethiae* inoculation in the oats, toxin levels were below the LOD or LOQ at all storage conditions except at 0.98 a_w at 20 °C, with HT-2-Glc concentrations of 37 ng/g. A similar trend was observed in the irradiated oat control samples.

In the irradiated inoculated oats, HT-2-Glc levels significantly increased with a rise in water activity and decreased with temperature rise, with concentrations higher than other oat treatments. HT-2-Glc was highest at 0.98 a_w at 20 °C. A similar trend was observed for the concentrations of HT-2 toxin in most storage conditions except at 0.98 a_w at 25 °C, where an increase in concentrations was observed, with the highest concentrations of 7167 ng/g. T-2 concentrations were significantly higher than HT-2 and HT-2-Glc concentrations at 0.95 a_w at both temperatures. However, at 0.98 a_w and at both 20 and 25 °C, T-2 concentrations were not significantly different ($p > 0.05$) from HT-2-Glc concentrations. HT-2 levels were significantly higher than T-2 concentrations at 0.98 a_w and 25 °C, and

no significant differences ($p > 0.05$) exist in their concentrations at 0.98 a_w and 20 °C, as shown in Table 3-3.

The p values of the statistical differences in the concentrations of T-2, HT-2, and HT-2-Glc under different storage conditions in all oat treatments are shown in Table C-2 and Table C-3 in Appendix C.

Table 3-3: Influence of a_w and temperature on the concentrations of T-2, HT-2, and HT-2-Glc in oats.

Mycotoxins (ng/g of grains) ^a						
Treatments	T (°C)	a_w	T-2 ¹	HT-2 ²	HT-2-Glc ³	Sum
Naturally contaminated oat control	20	0.95	<LOQ	26	41	67
		0.98	<LOD	<LOD	<LOQ	-
	25	0.95	<LOD	<LOD	<LOD	-
		0.98	<LOD	<LOD	<LOQ	-
Naturally contaminated oat + <i>F. langsethiae</i>	20	0.95	<LOQ	<LOQ	<LOQ	-
		0.98	<LOQ	<LOQ	37	37
	25	0.95	<LOD	<LOQ	<LOD	-
		0.98	<LOD	<LOQ	<LOQ	-
Irradiated oat control	20	0.95	<LOQ	20	<LOD	20
		0.98	<LOQ	<LOQ	<LOD	-
	25	0.95	<LOQ	<LOQ	<LOQ	-
		0.98	<LOQ	<LOQ	<LOD	-
Irradiated oat + <i>F. langsethiae</i>	20	0.95	4488 *	1655 *	704 *	6847
		0.98	7152	6037	5925	19,114
	25	0.95	1822 *	645 *	226 *	2693
		0.98	4117	7167	3690	14,974

^aResults are a mean of 4 replicates in different storage conditions. a_w —water activity. T—temperature. ¹T-2 toxin, ²HT-2 toxin, ³HT-2-glucoside. * = significant differences in the concentrations of T-2 and HT-2 at the same a_w (for the same temperature in each row). < LOD—below the limit of detection. <LOQ—below the limit of quantitation. Sum—T-2 + HT-2 + HT-2-Glc.

3.3.6 The effect of the interactions of water activity, temperature, and treatments on the percentage concentration ratios of HT-2-Glucoside.

The effect of the a_w , temperature, and the combined interactions of the storage conditions and the different treatments on the concentration ratios (%) of HT-2-Glc are significant, except for the interaction between treatment and temperature, as well as treatment, a_w , and temperature, as shown in Figure 3-3 below. The different letters of the Tukey HSD test show significant differences in the HT-2-Glc concentration ratios between both water activities at each temperature for all treatments. The HT-2-Glc concentration ratios in both irradiated treatments (IOC and IOFL) at 0.95 a_w at 20 °C were significantly different from other treatments, while at 25 °C, HT-2-Glc concentration ratios in the irradiated inoculated oats (IOFL) at 0.95 a_w were significantly different from other treatments.

The percentage concentration ratios of HT-2-Glc were calculated using Equation 3-4.

$$\text{HT-2-Glc \% ratio} = \frac{\text{HT-2-Glc}}{\text{T-2} + \text{HT-2} + \text{HT-2-Glc}} \times 100 \quad (3-4)$$

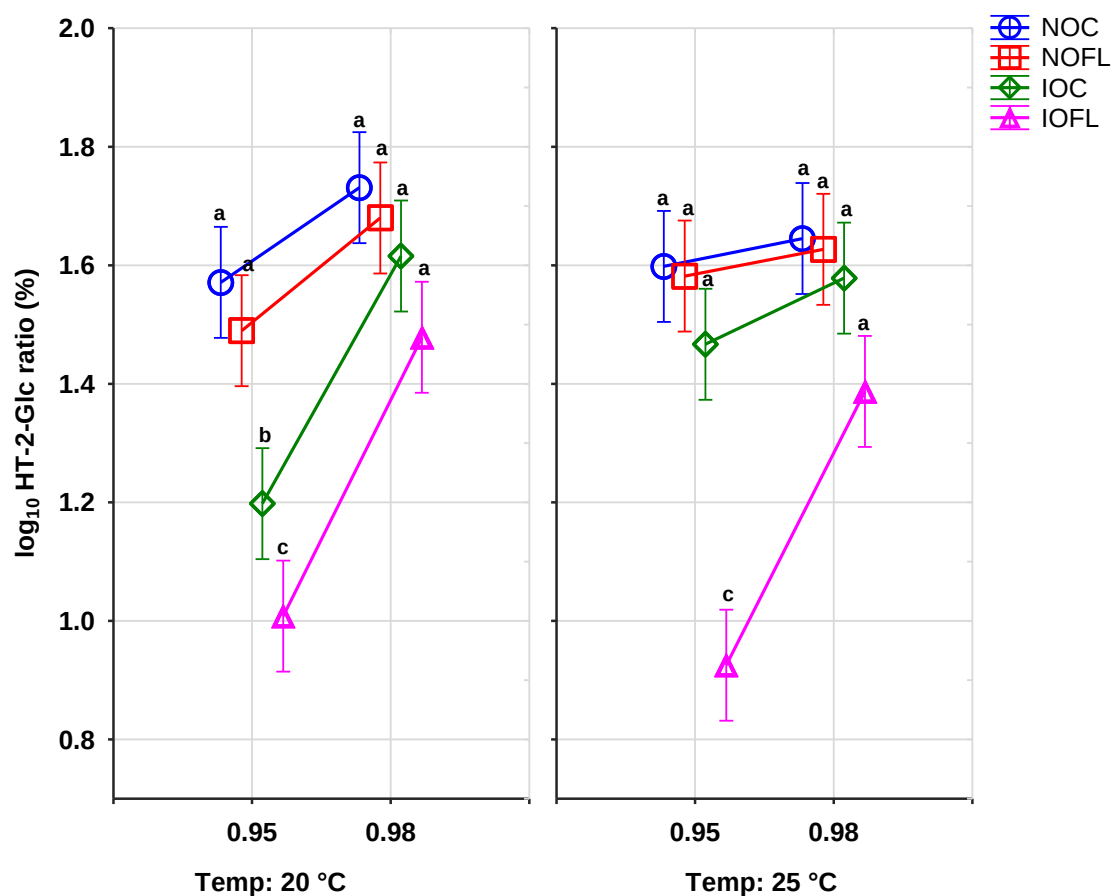


Figure 3-3: HT-2-Glc concentration ratios (%) in all oat treatments and all storage conditions. Vertical bars denote 0.95 confidence intervals. Different letters show significant differences in HT-2-Glc concentration ratios between all oat treatments at each water activity level for each temperature using the Tukey HSD test. HT-2-Glc: HT-2-glucoside. Water activity: 0.95 and 0.98. Temperature: 20 and 25 °C. NOC: naturally contaminated oat control. NOFL: naturally contaminated oat + *F. langsethiae*. IOC: irradiated oat control. IOFL: irradiated oat + *F. langsethiae*. Concentrations of analytes <LOD and <LOQ are assigned with values of LOD/2 and LOQ/2, respectively.

3.3.7 Impact of water activity, temperature, and treatments on DAS concentrations

The interactions between a_w , T, and treatment have no significant impact on the concentrations (ng/g) of DAS. However, water activity and treatment as a single factor had a significant effect on DAS concentrations, as shown in Figure 3-4 and b. Different letters of the Tukey HSD test show significant differences in DAS concentrations between water activities and in each treatment. DAS concentrations decreased significantly as water activities increased from 0.95 a_w to 0.98 a_w in all treatments. Its concentrations were highest at 0.95 a_w at 20 °C for both natural treatments. Although there was a decrease in DAS concentrations with temperature rise, the effect of temperature was not significant in all treatments. Also, DAS concentrations in all treatments and all conditions were not significantly different, except for concentrations in the irradiated inoculated oats (IOFL), as shown in Figure 3-4 b. The mean concentration values of DAS in all treatments and conditions are shown in Table **C-4** in Appendix C.

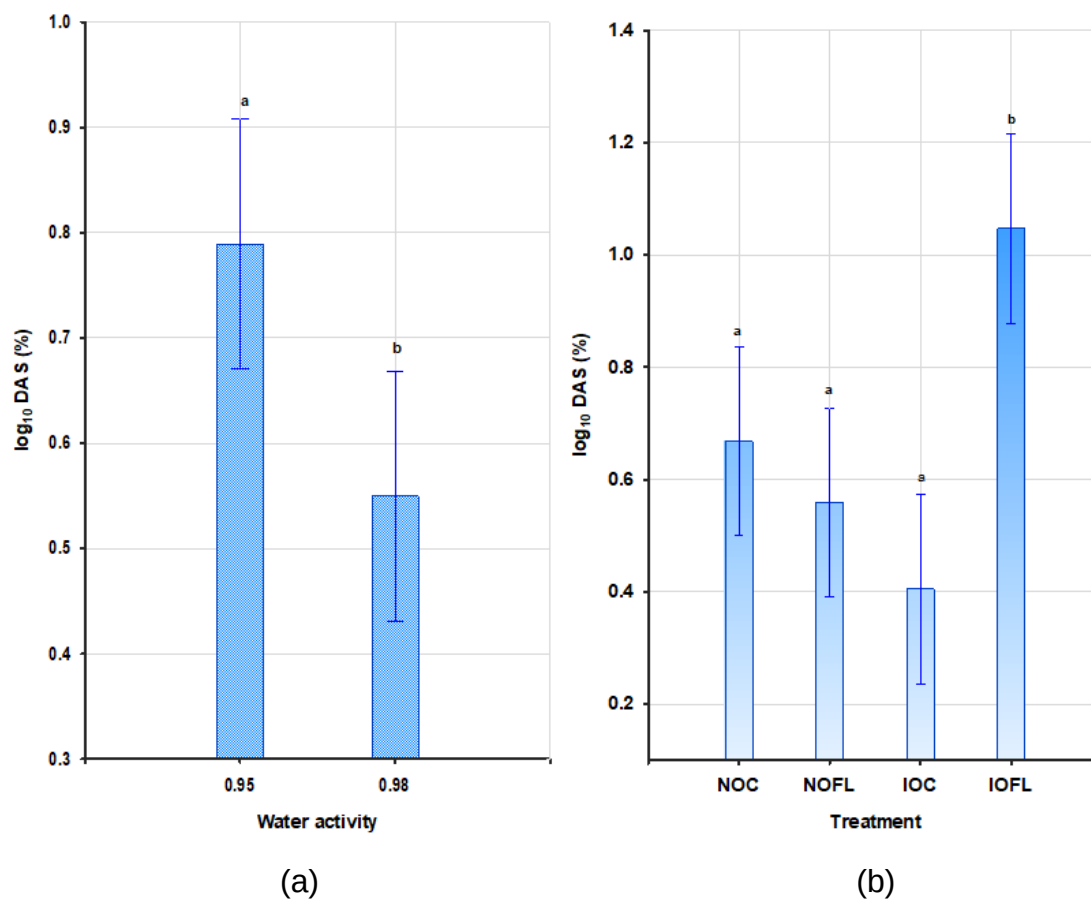


Figure 3-4: Impact of (a) water activity and (b) treatment on DAS concentrations (ng/g). Vertical bars denote 0.95 confidence intervals. Different letters show significant differences in DAS concentrations between both water activities and among the treatments using the Tukey HSD test. DAS: diacetoxyscirpenol. NOC: naturally contaminated oat control. NOFL: naturally contaminated oats + *F. langsethiae*. IOC: irradiated oat control. IOFL: irradiated oat + *F. langsethiae*. Concentrations of analytes <LOD and <LOQ are assigned with values of LOD/2 and LOQ/2, respectively

3.3.8 Impact of a_w and temperature on DON and DON-3-G in both natural and irradiated oat grains with and without *Fusarium langsethiae* inoculation

In the naturally contaminated oat control samples at 0.98 a_w and both temperatures, the levels of DON-3-G were about 2.7 times lower than the DON levels. It was present at levels high enough to increase the total DON content in the oats, exceeding the legislative limits. Surprisingly, DON-3-G concentrations at 0.98 a_w and 20 °C had a high mean value of 1869 µg/kg, which, in context, is higher than the maximum limits for DON. DON and DON-3-G concentrations increased with the rise in a_w but decreased with the temperature rise. However, significant differences in concentrations were only observed at 0.98 a_w and 25 °C, as shown in Table 3-4 below.

Meanwhile, in the naturally contaminated oats + *F. langsethiae*, DON-3-G and DON concentrations significantly increased with a rise in water activity. Also, there was a significant increase in DON-3-G concentrations with an increase in temperature at 0.98 a_w . In both irradiated oat treatments, there were no significant differences in the concentrations of DON-3-G and DON as the water activity and temperature increased Table 3-4.

The p values of the statistical differences in the concentrations of DON-3-G/DON under different storage conditions in all oat treatments are shown in Table C-5 in Appendix C.

Table 3-4: Influence of a_w and temperature on the concentrations of deoxynivalenol and DON-3-glucoside in oats.

Mycotoxins (ng/g of grains) ^a					
Treatments	T (°C)	a_w	DON ¹	DON-3-Glc ²	Sum
Naturally contaminated oat control	20	0.95	72	18	90
		0.98	5137	1869	7006
	25	0.95	27	6	33
		0.98	2212 *	818 *	3030
Naturally contaminated oat + <i>F. langsethiae</i>	20	0.95	1141 *	58 *	1199
		0.98	3995 *	1061 *	5056
	25	0.95	1636 *	64 *	1700
		0.98	5052 *	2297 *	7349
Irradiated oat control	20	0.95	<LOD	<LOD	<LOD
		0.98	21 *	7 *	28
	25	0.95	<LOD	<LOD	<LOD
		0.98	33 *	8 *	41
Irradiated oat + <i>F. langsethiae</i>	20	0.95	20	<LOQ	20
		0.98	55 *	7 *	62
	25	0.95	14	<LOQ	14
		0.98	16 *	7 *	23

^aResults are a mean of 4 replicates in different storage conditions. a_w —water activity. T—temperature. 1 Deoxynivalenol, 2 Deoxynivalenol-3-Glucoside. * = significant differences in the concentrations of DON and DON-3-G at the same a_w (for the same temperature in each row). <LOD—below limit of detection. <LOQ—below limit of quantitation. Sum—DON + DON-3-G

3.3.9 Impact of a_w and temperature on the concentrations of other secondary metabolites and emerging mycotoxins in natural and irradiated oat grains with and without *Fusarium langsethiae* inoculation.

In the naturally contaminated oat control samples, the highest concentrations of nivalenol were observed at 0.95 a_w at 25 °C, with a mean value of 230 ng/g. Also, 3-acetyl deoxynivalenol (3-AcDON) and 15-acetyl deoxynivalenol (15-AcDON) concentrations increased as water activity increased but decreased with the temperature rise. The enniatin (A, A₁, B, B₁), beauvericin (BEA), and moniliformin (MON) concentrations mostly increased with a rise in water activity and temperature, with all mycotoxins having the highest concentrations at 0.98 a_w at 25 °C.

In the naturally contaminated oats + *F. langsethiae*, the enniatins were below the limit of quantitation (LOQ) at 0.95 a_w , but their concentrations almost doubled at 0.98 a_w with the temperature rise. Generally, the concentrations of most emerging mycotoxins fell below the LOD and LOQ in the irradiated oat control and irradiated inoculated oats, respectively (Table 3-5).

Table 3-5: Influence of a_w and temperature on the concentrations of other secondary metabolites and emerging mycotoxins in oats

Mycotoxins (ng/g of Grains) ^a											
Treatments	T (°C)	a_w	3-AcDON	15-AcDON	NIV	ENN A	ENN A1	ENN B	ENN B1	MON	BEA
Naturally contaminated oat control	20	0.95	27	29	137	<LOQ	<LOQ	19	13	9	29
		0.98	89	126	138	8	56	251	192	205	142
	25	0.95	10	29	230	<LOQ	6	19	17	63	51
		0.98	26	89	90	25	190	539	461	3050	834
Naturally contaminated oat + <i>F. langsethiae</i>	20	0.95	9	62	237	<LOQ	<LOQ	<LOQ	<LOQ	13	23
		0.98	26	192	860	<LOQ	9	45	29	<LOQ	141
	25	0.95	21	93	206	<LOQ	<LOQ	<LOQ	<LOQ	19	22
		0.98	92	172	610	<LOQ	20	88	62	21	142
Irradiated oat control	20	0.95	<LOD	12	38	<LOD	<LOD	<LOD	13	<LOD	4
		0.98	<LOD	15	42	<LOD	<LOD	<LOD	13	<LOD	12
	25	0.95	<LOD	27	55	<LOD	<LOD	<LOD	24	<LOD	15
		0.98	<LOD	33	60	<LOD	<LOD	<LOD	21	<LOD	22
Irradiated oat + <i>F. langsethiae</i>	20	0.95	<LOQ	44	70	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	25
		0.98	<LOQ	68	90	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	47
	25	0.95	<LOQ	47	147	<LOQ	<LOQ	<LOQ	12	<LOD	36
		0.98	<LOQ	81	<LOQ	<LOQ	<LOQ	<LOQ	12	<LOD	39

^aResults are a mean of 4 replicates in different storage conditions. a_w —water activity. T—temperature. AcDON—Acetyldeoxynivalenol. NIV—Nivalenol. ENN—Enniatin. MON—Moniliformin. BEA—Beauvericin. <LOD—below limit of detection. <LOQ—below limit of quantitation.

3.4 Discussion

There is limited information available on the levels of T-2, HT-2, and HT-2-Glc in oats that are subjected to various storage conditions. This study aims to provide a detailed report on the concentrations of T-2, HT-2, HT-2-Glc, DON, DON-3-glucoside, and DAS (for the first time) in naturally and *Fusarium langsethiae* contaminated oats under different environmental conditions. The chosen storage conditions were 0.95 and 0.98 a_w at temperatures of 20 and 25 °C, which are conducive to the growth of *Fusarium* species and mycotoxin contamination in oat grains. These temperatures are similar to those found in the European climate [36]. Previous research has indicated that *Fusarium langsethiae* may colonize oat grains before and after flowering due to specific environmental factors, leading to the accumulation of HT-2 and T-2 [37]. Values of 0.95–0.98 a_w may represent a moisture content between 25 and 28% [38] that can be achieved in oat grains post-harvest due to inefficient drying or compromised storage conditions resulting from pest infestations, silo leaks, seasonal rainfall, or temperature changes [39].

The influence of a_w and temperature on the levels of HT-2-Glc and HT-2 toxin in naturally contaminated oats (control samples) was insignificant. However, the highest concentrations of these toxins were observed at 0.95 a_w and 20 °C, indicating that *Fusarium langsethiae* was able to grow well in grains under these conditions. This finding contrasts with a previous study which reported that the highest growth range for *F. langsethiae* in vitro (oat-based medium) occurred at 0.98–0.995 a_w and 25 °C [31]. It is understood that *F. langsethiae* will continue to thrive under cool and damp conditions. Interestingly, under the same storage conditions (0.95 a_w and 20 °C) and treatment (naturally contaminated oats control), the concentration of HT-2-Glc was approximately 1.5 times higher than that of HT-2. The levels of T-2 were below the LOQ compared to its acetylated form, HT-2 toxin. Other studies have also reported higher concentrations of HT-2 than its precursor, T-2 toxin [2,4]. Daud et al. [40] reported that concentrations of HT-2 toxins were higher than T-2 toxins in all conventional and organic Scottish oats examined. However, the interactions between different abiotic factors were not considered in these previous studies. Medina and Magan [41] reported higher

concentrations of T-2 toxin than HT-2 toxin at 0.95 a_w and 20 °C produced by *F. langsethiae* strains isolated from English oats grown on an oat-based medium. The contrast to the T-2 concentrations reported in our study could be due to the deacetylation of T-2 toxin to HT-2 toxin by the oat grains, which did not occur in the oat-based media. Since studies on the metabolic pathways of HT-2 in *F. langsethiae* are limited, there are still uncertainties about whether the high production of HT-2 was mainly due to the strain of fungus present or the natural mycobiota rapidly hydrolysing T-2 to HT-2 due to ecological stress, or the cereal itself as reported in other studies by Lattanzio et al. [42] and Kokkonen et al. [43]. Weather conditions in different geographical locations could also be responsible for differences in toxin contamination [37,44].

As the population of *Fusarium langsethiae* increased due to inoculation, the concentrations of T-2 and HT-2 were found to be below the LOD or LOQ under all storage conditions. This indicates that the growth and toxin production of *F. langsethiae* may be affected by other competitive fungi that are present in the oat grains.

The irradiated inoculated oats had a higher concentration of *F. langsethiae* spores (10^6 spores/mL) compared to the naturally contaminated oats + *F. langsethiae* samples. This was due to the absence of or lesser competition from other natural fungi that compete for space and nutrients. The concentrations of T-2, HT-2, and HT-2-Glc were high in the irradiated inoculated oat grains and exceeded the recommended levels set by the European Union under all storage conditions. This demonstrates the *F. langsethiae* strain's ability to produce T-2, HT-2, and its glucoside HT-2-Glc under both water and temperature stress. The concentrations of T-2, HT-2, and HT-2-Glc were significantly different in most storage conditions, except at 0.98 a_w and 20 °C.

Generally, the highest concentrations of HT-2-Glc were observed at 0.98 a_w and 20 °C in both irradiated oat treatments. A similar observation was made by Mylona and Magan [32], who reported the highest concentrations of T-2 and HT-2 toxins in irradiated oat samples at a temperature of 25 °C and a water activity of 0.97 a_w after ten days of storage. The study revealed that water activity had a

greater impact on toxin production than temperature. This finding supports our observations in the irradiated oat grains.

Although there are currently no legal or recommended limits for diacetoxyscirpenol (DAS) in oats, the naturally contaminated oat control samples showed the highest concentrations of DAS at 0.95 a_w and a temperature of 20 °C, with a mean value of 12 µg/kg. However, DAS levels decreased in the naturally contaminated oats + *F. langsethiae*, despite the fungal inoculation. The highest concentrations of DAS were observed at 0.95 a_w and a temperature of 20 °C, with a mean value of 7 µg/kg. On the other hand, the irradiated oats under the same storage conditions had a mean value of 19 µg/kg, as shown in

Table C-4 in Appendix C. These findings suggest 20 °C as the optimum temperature for DAS production. Bryła et al. [45] analysed DAS alongside other trichothecenes in oats and reported its concentrations to be lower than the limit of quantitation (LOQ) of 1 µg/kg, which was lower than the LOQ in our study. Also, it is important to note that the study did not consider DAS under different environmental conditions.

As a regulated mycotoxin, it is important to analyse DON concentrations alongside T-2 and HT-2 toxins in oats. Our findings show that the concentrations of DON were higher than T-2 and HT-2 toxin concentrations in the naturally contaminated oats. This agrees with the observations of Nathanail et al. [46]. Their study reported DON and its glucoside, DON-3-G, with mean values of 2690 µg/kg and 806 µg/kg, respectively, while T-2, HT-2, and its glucoside HT-2-Glc had mean concentrations of 60.1 µg/kg, 159 µg/kg, and 41.4 µg/kg, respectively. However, the Finnish oat samples used had a moisture content below 15% (<0.70 a_w) compared to the wetter conditions used in our study. These studies suggest that *Fusarium graminearum* might have a growth advantage over *Fusarium langsethiae* under the conditions studied, leading to an increase in the production of DON in the oats, with the occurrence of DON in oats reported by other studies [6,47].

In contrast to our findings, Langseth and Rundberget [2] reported higher concentrations of HT-2 (115 µg/kg) and T-2 (60 µg/kg) than DON concentrations, which were 56 µg/kg in Norwegian oats samples. The mean value of the moisture content of the Norwegian oats was 15 (±2). In contrast to the naturally contaminated oat control samples, DON concentrations in the irradiated oats were relatively low due to the increased concentrations of T-2 and HT-2 toxins produced by *F. langsethiae*.

Surprisingly, DON and DON-3-G concentrations were found in the irradiated control samples in the wettest condition, 0.98 a_w , at both temperatures. This suggests that the moisture content plays a crucial role in the fungal contamination of grains.

In the naturally contaminated oat control samples, enniatin B had the highest mean concentrations (539 µg/kg) among all enniatins in all storage conditions at 0.98 a_w at 25 °C. The occurrence of enniatin B at the highest concentrations among all the enniatins studied was also reported by Uhlig et al. [48]. The mean concentrations of enniatin B in their study were 47 µg/kg, 490 µg/kg, and 790 µg/kg in Norwegian oats, barley, and wheat samples, respectively. However, the grains analysed did not have the same storage conditions as those used in our study. In the wettest and warmest storage conditions, MON and BEA had their highest mean concentrations of 3050 µg/kg and 834 µg/kg, respectively. However, in the naturally contaminated oats + *F. langsethiae*, MON, and BEA concentrations were remarkably lower at the same storage conditions (Table 3-5).

3.5 Conclusion

This research shows that the levels of *Fusarium* toxins vary based on different storage conditions. As water activity changes, conjugated mycotoxins co-occur with precursor toxins, secondary metabolites, and emerging toxins in stored oats. Although the amount of T-2, HT-2, and HT-2-Glc in the naturally contaminated oats was not higher than the recommended or indicative levels, the coexistence

of DON-3-G with DON was significant, leading to an increase in the total deoxynivalenol content and thus increasing the overall toxicity of the oats.

Mycotoxin levels in the inoculated grains indicated a high risk of contamination. This risk could occur due to inefficient drying regimes or changes in the storage environment, especially if the populations of *Fusarium langsethiae* are high in oats. These findings highlight the importance of conducting a robust analysis of mycotoxins in grains to avoid underestimating the total toxin content. Furthermore, since the toxicity of T-2 and HT-2 is similar [49,50] and our findings showed no significant differences in their concentrations, it is important to efficiently dry grains after harvest and monitor the abiotic conditions of the storage environment. This will help to reduce high concentrations of either individual or sums of trichothecene toxins and their respective conjugates in oat grains

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4 The influence of different abiotic conditions on the concentrations of free and conjugated deoxynivalenol and zearalenone in stored wheat.

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Abstract

Environmental factors influence fungal growth and mycotoxin production in stored grains. However, the concentrations of free mycotoxins and their conjugates and how they are impacted by different interacting environment conditions have not been previously examined. The objectives of this study were to examine the impact of storage conditions (0.93–0.98 a_w) and temperature (20–25 °C) on (a) the concentrations of deoxynivalenol and zearalenone and their respective glucosides/conjugates and (b) the concentrations of emerging mycotoxins in both naturally contaminated and irradiated wheat grains inoculated with *Fusarium graminearum*. Contaminated samples were analysed for multiple mycotoxins using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Method validation was performed according to the acceptable performance criteria set and updated by the European Commission regulations No. 2021/808/EC. As an important conjugate of deoxynivalenol, the concentrations of deoxynivalenol-3-glucoside were significantly different from its precursor deoxynivalenol at 0.93 a_w (22% moisture content- MC) at 25 °C in the naturally contaminated wheat with a ratio of 56:44, respectively. The high concentrations of deoxynivalenol-3-glucoside could be influenced by the wheat's variety and/or harvested season/fungal strain type/location.

Zearalenone-14-sulphate concentrations were surprisingly three times higher than Zearalenone in the naturally contaminated wheat at 0.98 a_w (26% MC) at both temperatures. Emerging mycotoxins such as moniliformin, increased with temperature rise with the highest concentrations at 0.95 a_w and 25 °C. These findings highlight the influence and importance of storage a_w x temperature conditions on the relative presence of free vs conjugated mycotoxins which can have implications for food safety.

Keywords: Deoxynivalenol, zearalenone, conjugated mycotoxins, water activity, and LC-MS/MS.

Key contribution: This study shows that interacting environmental factors of a_w and temperature of stored wheat influence the concentrations of deoxynivalenol (DON) and zearalenone (ZEN) and their respective modified forms. Current legislation is based on free DON and ZEN, and perhaps the relative importance of their conjugated mycotoxins needs to be considered in the future.

4.1 Introduction

Wheat grains are an important staple food consumed worldwide. They are used as animal feeds, and ingredients in food processing, or brewing industries [1]. However, pre- and post-harvest colonisation by mycotoxigenic fungi and mycotoxins can occur depending on pre-harvest weather conditions and whether effective drying regimes have been applied for safe storage [2]. *Fusarium graminearum* is predominantly responsible for *Fusarium* head blight (FHB) or ear blight during the ripening of wheat ears. This can result in contamination with deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEN) which pose safety concerns to human and animal health. They affect cereal grains' quality and yield with significant economic losses [3–5].

DON causes gastroenteritis in humans and immunotoxicity, impaired reproduction, and development in animals [6,7]. ZEN is a potent estrogenic metabolite causing hormonal imbalance in the body [8]. Owing to these safety concerns, strict legislation exists, regulating the maximum permitted concentrations of these mycotoxins in food and feed for human and animal use [9]. However, these regulations are based on the free mycotoxins and do not include conjugated mycotoxins, which are generally considered to be 'less toxic' but can be converted to toxic forms within the digestive tract of mammals [10].

As part of plants' defence mechanism against infection by fungi such as *F. graminearum*, it metabolizes some of the mycotoxins produced by the fungi through glucosylation to produce other forms of mycotoxins generally referred to as 'conjugated mycotoxins' [11],

For example, a cytoplasmatic UDP-glycosyltransferase was isolated and characterised from the plant *Arabidopsis thaliana* capable of transforming DON into DON-3- β -D-glucopyranoside (DON-3-glucoside) where a glucose unit replaced the hydroxyl group in position C₁₃ of DON [12]. The glucosylated mycotoxins can be stored in plant vacuoles, where they are protected against cleavage by β -glucosidases to prevent hydrolysis of the β -glucosidic bonds.

Berthiller et al. [10] demonstrated that DON-3-glucoside is not affected by the acidic condition in the stomach, however, there is evidence that human lactic acid bacteria can cleave the glucoside to release DON, hence reactivating its toxicity.

Conjugated mycotoxins are not easily detected in routine analysis as they may not be extracted by the solvents used, or they may be lost during the clean-up process due to their polarity or different chemical structures [13,14]. Conjugated mycotoxins have gained the attention of scientific research in recent times because they co-occur with their precursors in food and feeds, thereby increasing exposure [15]. When compared to estimates made with conventional analytical approaches, there could be an underestimation of the total toxin content of contaminated food or feed, therefore, advanced analytical methods such as LC-MS/MS have been developed to detect conjugated mycotoxins in different food matrices and standards for their analytical detection are becoming commercially available [13,16].

Several studies have reported the co-occurrence of both free and conjugate - mycotoxins in food or feed samples [17–19]. Other studies showed the conversion of DON and ZEN to their respective glucosides in foods and feeds [20–22], these studies have not considered how storage conditions affect the relative ratios of free vs bound mycotoxins.

Streit et al. [23] reported the co-occurrence of DON-3-G found in 75% of samples while DON was present in 89% of the feed and feed ingredients samples analysed. ZEN-14-S co-occurred with ZEN in 75% and 87% of the samples respectively. However, no information was given about the percentage ratios of co-occurrence of the conjugated mycotoxins and their precursor toxins in each of the positive samples. Another study showed that ZEN sulphate co-occurred with ZEN with a mean value of 14 µg/kg and was present in 18% of maize samples while its parent toxin ZEN contaminated 36% of maize samples with mean values of 13.6 µg/kg. No other ZEN metabolites were analysed in the study [19]. Owing to this, toxicity assessments for all mycotoxin derivatives present in food and feeds are essential to estimate the health risk posed by the sum of different forms of a specific mycotoxin [13].

It is important to stress that storage conditions are major factors that influence fungal growth and mycotoxin production in stored cereals [24,25]. Studies have documented the ecophysiology of different fungi and free mycotoxins under different storage conditions [2,26–28]. For example, the marginal and optimal conditions supporting the growth of *Fusarium graminearum* was reported as 0.90–0.995 a_w at 15–25 °C while DON production was observed at 0.93–0.995 a_w and 15–30 °C, although this sometimes depends on incubation time [26,29,30]. Kokkonen et al. [31] stated that warm temperatures (20–28 °C) and cooler temperatures (15 and 17°C) increased ZEN production by *F. graminearum* and *Fusarium culmorum*, respectively.

The objectives of this study were to examine the impact of storage conditions of water activities 0.93, 0.95, 0.98 a_w and temperature 20–25 °C on (a) the concentrations of DON and ZEN and their respective glucosides/conjugates and (b) the concentrations of emerging mycotoxins in both naturally contaminated and irradiated wheat grains inoculated with *Fusarium graminearum* to ascertain any potential increases in toxicity in the wheat grains.

4.2 Materials and Methodology

4.2.1 Fungal strain.

Fusarium graminearum Fg08/111 strain known to produce DON and ZEN was used in this study. Glycerol: water (70:30, v/v) was used to maintain the strain at -20 °C in the culture collection of the Magan Centre of Applied Mycology Cranfield University. *Fusarium graminearum* was grown on Malt Extract Agar (MEA) media prepared with chloramphenicol (an anti-bacterial agent) and was incubated at 25 °C for 7 days. For active sporulation, it was subcultured on V8 agar media for 7 days at 25 °C.

4.2.2 Wheat grains

Wheat grains were collected 3months after harvest from Bedfordshire farms (2020 harvest; variety: Belepi). Grains were stored at 4 °C five months before the experiment. 12.5–15 kGys doses (STERIS, Bradford, West Yorkshire) were used to disinfect 5kg of grains from fungal contaminants while retaining their germinating capacity. To check for the initial mycobiota (fungal isolation) of the natural and irradiated grains, 5 grains were placed equidistant on five MEA+ media on 9cm petri plates in a sterile flow bench and then incubated at 25 °C for 7 days. The fungal contamination was evaluated visually with a stereoscope (Olympus 308136, Wild M7A, Heerbrugg, Switzerland).

4.2.3 Moisture adsorption curve analysis

10 g sub-samples of natural or irradiated wheat grains were placed in ten 25 mL universal bottles. Known amounts of water (0.1–3.5 mL) were added to the grains. The bottles were sealed and mixed thoroughly and stored for 24 hours at 4 °C with consistent shaking. The samples were equilibrated at room temperature for an hour and the water activity of the grains was analysed (AquaLab water activity meter 4 TE Decagon devices, Inc, Pullman, USA). The grains were oven-dried overnight at 105 °C and checked for moisture content (MC) Three replicates

were analysed for each grain treatment. To achieve the targeted water activity levels for grain modification, a graph showing the amount of water added against a_w values was plotted (see Figure 4-1). The relationship between the MC (dry weight basis) and a_w values was also plotted and noted.

4.2.4 Grain Inoculation

Natural and Irradiated wheat grains (120 g) were modified with sterile water using the moisture adsorption curve above to achieve the targeted a_w levels of 0.93, 0.95, and 0.98 a_w and stored at 4 °C for 24 hours to equilibrate. 15 g of the equilibrated grains were placed in 40 mL Volatile Organic Analysis (VOA) vials with sealable polytetrafluoroethylene (PTFE) caps containing a silicone septum for gas exchange. Four 5 mm diameter agar discs of a 7-day old colony of *Fusarium graminearum* (Fg08/111) strain grown on MEA+ agar were added into each 15 g of grains in each glass vial of 4 replicates and thoroughly mixed. A control treatment without fungi inoculation was also prepared. Grains with the same a_w levels were placed in 12-litre poly-propylene environmental chambers with 2x 500 mL beakers of glycerol-water solution to maintain the target Equilibrium Relative Humidity (ERH) of the atmosphere for each a_w level. The glycerol-water solution was changed after every five days during storage. The chambers were stored at temperatures of 20 °C and 25 °C for 18 days. Subsequently, the grains were dried at 55 °C overnight, milled, and stored at –20 °C before further analysis.

4.2.5 Mycotoxin analysis

4.2.5.1 Chemical reagents and mycotoxin standards

The Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) grade methanol, acetonitrile, and formic acid (Honeywell, Seelze, Germany), ammonium acetate (MS grade, Sigma-Aldrich, Germany), and glacial acetic acid (Sigma-Aldrich, USA) were purchased. Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France). Conjugated mycotoxins standards used are DON-3-G purchased from Romer

labs (Tulln, Austria), and ZEN-14-S from Aokin AG (Germany). Other mycotoxin standards: DON, ZEN, 15- Acetyl-deoxynivalenol (15-AcDON), 3- Acetyl-deoxynivalenol (3-AcDON), zearalenone-14-glucoside (ZEN-14-G), zearalenone-16-glucoside (ZEN-16-G), diacetoxyscirpenol (DAS), enniatin A (ENN A), enniatin A₁ (ENN A₁), enniatin B (ENN B), enniatin B₁ (ENN B₁), moniliformin (MON) and nivalenol (NIV) were purchased from Romer Labs (Tulln, Austria) and supplied by the Institute for Global Food Security, Queen's University Belfast (QUB), UK. Individual stock solution was mostly prepared at 1 µg/mL in appropriate dilution solvent (Acetonitrile) in amber glass vials and stored at -20°C and allowed to equilibrate at room temperature before use.

4.2.5.2 Sample preparation and extraction.

The initial mycotoxin concentrations of the wheat grains were analysed after the reception. A total of 96 contaminated wheat samples (including replicates) were analysed for mycotoxins after the storage experiment. 4 mL of the extraction solvent (acetonitrile (ACN): water (H₂O): acetic acid (HAc) 79: 20:1, v/v/v) was added to 1 g of ground wheat grains. Samples were extracted for 90 minutes using a multitube vortex (VWR DVX-2500, VMR International Ltd Leicestershire, UK) and subsequently centrifuged at 5000 rpm for 15 minutes on a Rotina 380R centrifuge (Hettich, Tuttlingen, Germany). 200 µL of the extract was diluted with 800 µL dilution solvent (ACN: H₂O: HAc 20:79:1, v/v/v), yielding a 1:4 dilution. The aliquots were filtered into the LC-MS/MS vials through a 0.22 µm filter with a 2.5 mL syringe and vortexed. 5 µL of the diluted extracts were injected into a LQTRAP 5500+ MS/MS (LC-MS/MS) system (SCIEX, Framingham, MA, USA).

4.2.5.3 LC-MS/MS parameters

The chromatogram separation was performed using the SCIEX Exion LC™ AD system with detection via SCIEX triple Quad 5500+ QTrap LC-MS/MS system equipped with Turbo V™ ionisation source operated in both positive and negative mode. Detection and quantification were accomplished using targeted analysis

via a scheduled Multiple Reaction Monitoring. Separation was performed at 27 °C on a Gemini C18-column, 100 × 4.6 mm (Phenomenex, Torrance CA, USA).

Elution was done with a binary gradient mode consisting of mobile phase A—methanol/water/acetic acid 10:89:1 (v/v/v) and mobile phase B—methanol/water/acetic acid 97:2:1 (v/v/v), both containing 5 mM ammonium acetate buffer. The mobile phase flow rate was maintained at 0.8 mL/min, with the sample injection volume set at 5 µL, and the total runtime was 7.5 minutes. The gradient elution program for the elution of mycotoxins was as follows: 0 min 5%B, 0.5 min 5% B, 2.5 min 70% B, 3.5 min 95% B, 5 min 95% B, 7.5 min 5% B. Mass Spectrometric and chromatographic parameters are declustering potential (DP), collision cell exit potential (CXP), and collision energy (CE), as shown in Table 4-1. The chromatographic method, as well as chromatographic and mass spectrometric parameters, were adapted from Malachová et al. [16]

Table 4-1: Optimised MS/MS parameters for the analysed mycotoxins, including precursor ions, product ions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP).

Mycotoxins	Precursor ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)
Regulated mycotoxins					
Deoxynivalenol	297.1	249.1	91	21	20
	297.1	203.2	91	21	20
Zearalenone	317.1	131.1	-100	-42	-8
	317.1	175	-100	-34	-13
Masked Mycotoxins					
3-Acetyldeoxynivalenol	397.3	59.2	-60	-38	-8
	397.3	307.1	-60	-20	-7
15-Acetyldeoxynivalenol	339.1	321.3	81	13	18
	339.1	261.1	81	17	14
Deoxynivalenol-3-Glucoside	517.2	427.1	-115	-30	-11
	517.2	457.2	-115	-20	-19
Zearalenone-14-glucoside	479.2	317.1	-145	-24	-15
	479.2	175	-145	-54	-11
Zearalenone-16-Glucoside	479.1	317.1	-140	-30	-21
	479.1	149	-140	-50	-15
Zearalenone-14-Sulphate	397.1	317.1	-130	-32	-17
	397.1	175	-130	-48	-11
Emerging mycotoxins					
Diacetoxyscirpenol	384.2	307.3	86	17	16
	384.2	247.3	86	21	14
Enniatin A	699.4	682.4	10	27	24
	699.4	210.2	10	39	22
Enniatin B	657.3	640.3	10	27	22
	657.3	196.1	10	39	10
Enniatin B ₁	671.3	654.4	6	27	22
	671.3	196.1	6	41	22
Enniatin A ₁	685.4	668.5	11	27	12
	685.4	210.1	11	39	10
Moniliformin	96.9	41.2	-5	-38	-14
Nivalenol	371.1	281.1	-90	-20	-15
	371.1	59.1	-90	-50	-7

m/z: mass to charge ratio.

4.2.5.4 Optimised LC-MS/MS method validation

The optimised LC-MS/MS method for mycotoxins analysis in wheat was validated based on the acceptable performance criteria of analytical methods set and updated by the European Commission regulations No. 2021/808/EC [32]. The performance characteristics evaluated were linearity (r^2), limit of detection (LOD), limit of quantification (LOQ), selectivity, matrix effect, recovery, and repeatability. Extraction and apparent recoveries were determined by spiking homogenised blank wheat (purchased from Romer labs, Tulln, Austria) at three different levels with multi-mycotoxin working standards solution with concentrations of 0.5 µg/mL (for DON-3-G and the Enniatins) and at 1 µg/mL (for other toxins standards listed in session 4.2.5.1 above) depending on the analytes. Spiking levels for each analyte are shown in Table **D-1** in Appendix D.

Quantification was performed via external calibration using nine serial dilutions of a multi-analyte stock solution ranging from calibration 1 (500 µL/mL) to calibration 9 (0.1 µL/mL). According to Sulyok et al. [33], the recoveries of the extraction and matrix effects have not been found to differ between different concentration levels (covering up to a factor of 1000). Data were further processed using Analyst® Software 1.7.1 and SCIEX OS-Q Software. The validation procedure was adapted from Siri-Anusornsak et al. [34].

4.2.6 Statistical analyses

The data sets were analysed with JMP® Pro 16, Microsoft Excel, and Statistica 14.0.1. Shapiro-Wilk and Levene's tests were used to test for data normality and homoscedasticity respectively. Data transformation was done where data sets failed the normality test. Transformed data were normally distributed therefore one-way ANOVA was used to find differences between groups. Differences among each toxin pair for each treatment were analysed using nonparametric comparisons for each pair Wilcoxon method. The significant impact of the interactions of the storage conditions and treatments on the DON-3-Glc and ZEN-14-Sulphate concentration ratios was analysed with a Tukey HSD test. The

normal plots of the residuals were used to examine the linearity assumption and the normal distribution of the residuals. Statistical analyses performed were considered significant when the p -value < 0.05 . Concentrations of analytes $< \text{LOD}$ and $< \text{LOQ}$ are assigned with values of $\text{LOD}/2$ and $\text{LOQ}/2$, respectively [35].

4.3 Results

4.3.1 Moisture adsorption curve

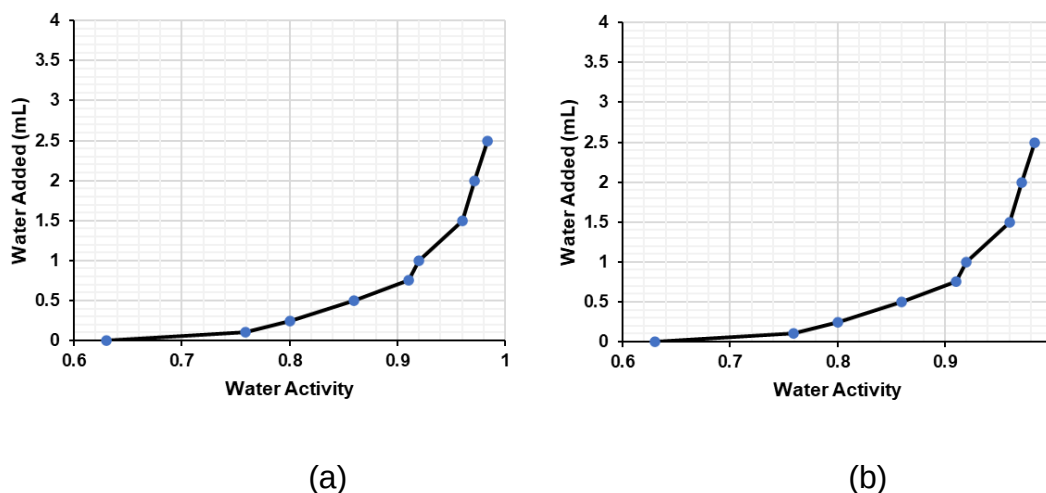


Figure 4-1: Moisture Adsorption Curves for 10 g of (a) natural wheat (d) irradiated wheat grains.

The MAC graph is used to calculate the equivalent amount of water from their respective a_w level which is then modified for grain rehydration depending on the weight of the grain.

4.3.2 Method validation performance in the wheat matrix.

The extraction efficiency (R_E) varies with each analyte and was within the acceptable range (70–120%) according to the amended guideline set by the European Commission regulations No. 2021/808/EC [32] except for Moniliformin which was slightly out of range. The goodness of fit of the calibration curve for

each analyte has good linearity with r^2 (coefficient of determination) > 0.990 . The trueness and precision represented by the relative standard deviation (RSD%) of the method were satisfactory for the validated analytes. The RSD was $<11\%$ for both the repeatability and reproducibility of the method. The range of the limit of detection (4–12 $\mu\text{g/kg}$) and the limit of quantitation (14–39 $\mu\text{g/kg}$) for the analysed analytes were lower than the minimum acceptable levels for the regulated mycotoxins in unprocessed wheat [9]. The validation performances for each analyte are shown in Table 4-2. The validation was performed by spiking 6 replicates of wheat at 3 different concentrations on 3 separate days. Therefore, the measurement uncertainties were calculated using the lowest level -Level 1 as shown in Table D-1 in Appendix D, averaging all 18 replicates to get the standard deviation, which was multiplied by 2 or 3 as shown in Table D-2 in Appendix D.

Table 4-2: Mean values (n=9) of the inter-day precision within lab reproducibility (WLR) method validation for wheat grains.

Components	RSD	R _A	R _E	LOD (ng/g)	LOQ (ng/g)
Moniliformin	3.4%	72.3%	62%	5	17
15-Acetyldeoxynivalenol	4.0%	77.0%	90%	5	18
3-Acetyldeoxynivalenol	4.5%	68.7%	93%	4	14
Diacetoxyscirpenol	3.7%	101.3%	95%	4	13
Deoxynivalenol	3.6%	98.7%	98%	12	39
DON-3-Glucoside	3.8%	92.3%	83%	6	20
Enniatin A	2.5%	86.8%	97%	4	14
Enniatin A1	3.6%	97.7%	97%	6	21
Enniatin B	4.7%	104.0%	96%	11	36
Enniatin B1	3.6%	100.7%	96%	7	23
Nivalenol	5.4%	98.9%	90%	10	35
Zearalenone	3.3%	96.5%	99%	8	27
ZEN-14-G	3.4%	107.4%	92%	5	17
ZEN-14-S	6.4%	34.3%	90%	4	14
ZEN-16-G	3.5%	111.5%	96%	7	22

RA- apparent recovery, RE- extraction efficiency, RSD – relative standard deviation, LOD- limit of detection,

LOQ- limit of quantitation.

4.3.3 The initial fungal isolation, deoxynivalenol, its glucoside DON-3-G, and ZEN concentrations of wheat grains before storage experiment.

There was 100% fungal isolation from the 25 grains analysed in the naturally contaminated wheat while no growth occurred in the 25 grains analysed for the irradiated grains on the malt extract agar (MEA+) media. Pictorial representations of the contaminated plates are in Figure 4-2.

One-way ANOVA shows that the initial average concentrations of DON-3-G (27.8 ng/g) were significantly higher than DON's concentrations (18.8 ng/g) in the naturally contaminated wheat $p= 0.0438$. Zearalenone's concentrations were below the limit of detection. The mean values ($n=3$) of the grains' water activity and moisture content before the storage experiment are 0.67 a_w and 13.3% respectively.

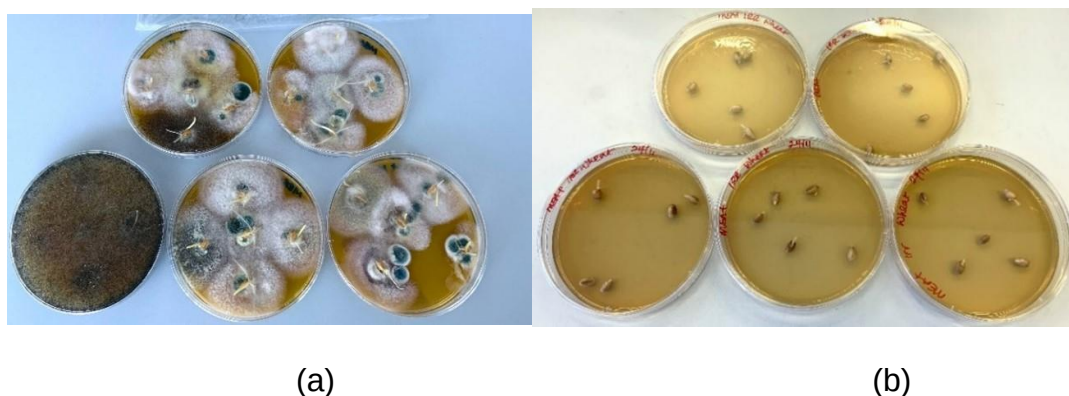


Figure 4-2: Pictorial representations of the Fungal isolations of the initial microbiota in (a) naturally contaminated wheat grains (b) irradiated wheat grains on Malt Extract Agar (MEA+). +: chloramphenicol

4.3.4 Water activity and temperature impact on the concentrations of DON and DON-3-glucoside in naturally contaminated wheat grains with and without *Fusarium graminearum* inoculation.

In the naturally contaminated wheat control samples at 0.93 a_w , the concentrations of DON-3-G were at least 1.2 times higher than DON concentrations with significant differences in their concentrations at 25 °C. There are no significant differences ($p > 0.05$) in the concentrations of DON-3-G and DON at the wettest condition (0.98 a_w) at both temperatures. DON concentrations significantly increased with the rise in water activity, but temperature rise had no significant impact on its concentrations. At all storage conditions, DON content did not exceed the maximum limit (Table 4-3).

In the naturally contaminated inoculated wheat with *F. graminearum*, there was a significant rise in the concentrations of DON-3-G from 0.95–0.98 a_w at both temperatures with the highest concentrations of DON-3-G at 0.98 a_w and 25 °C. The concentrations of DON compared to its glucoside DON-3-G were significantly high at all storage conditions. Due to inoculum load, DON content exceeded the maximum limit at 0.98 a_w for both temperatures compared to the naturally contaminated wheat.

4.3.5 Water activity and temperature impact on the concentrations of DON and DON-3-glucoside in irradiated wheat grains with and without *Fusarium graminearum* inoculation.

In the irradiated control wheat, there were no measurable DON or DON conjugates at all storage conditions. The concentrations of DON-3-G were significantly lower than DON in the inoculated irradiated wheat with *Fusarium graminearum* at all storage conditions except at 0.93 a_w , 25 °C as shown in Table 4-4.

Table **D-4** and Table **D-5** in Appendix D show the impact of each storage condition on the concentrations of DON-3-G and DON and the statistical differences in the concentration ratios of DON-3-G to DON in all treatments.

It is important to note that the effect of the interaction of a_w x temperature x treatment was significant on the concentration ratios of DON-3-G. However, temperature as a single storage factor and its interaction with water activity (temp x a_w) did not significantly impact the concentration ratios of DON-3-G (Figure **D-1** in Appendix D).

Table 4-3: Mean values of concentrations (ng/g) of Deoxynivalenol, its DON-3-glucoside and other derivatives in naturally contaminated wheat $\pm$ *F. graminearum* (n=4) at different storage conditions.

Naturally contaminated wheat control							Natural wheat + <i>F. graminearum</i>				
T (°C)	a _w	DON ¹	DON-3-G ² (ng/g)	3-AcDON ³ (ng/g)	15-AcDON ⁴ (ng/g)	Sum (ng/g)	DON ¹ (ng/g)	DON-3-G ² (ng/g)	3-AcDON ³ (ng/g)	15-AcDON ⁴ (ng/g)	Sum (ng/g)
20	0.93	64	83	<LOD	55	202	111	31*	<LOD	62	204
	0.95	82	36*	<LOQ	58	176	99	<LOD	<LOQ	46	145
	0.98	296	94	21	<LOD	410	4096	202*	74	35	4407
25	0.93	53	91*	<LOD	<LOD	144	89	<LOD	<LOD	<LOD	89
	0.95	69	43*	<LOD	31	143	198	31*	<LOQ	<LOD	228
	0.98	181	97	26	27	331	4726	284*	26	30	5066

¹ Deoxynivalenol, ² Deoxynivalenol-3-Glucoside, ³ 3-acetyldeoxynivalenol, ⁴ 15-acetyldeoxynivalenol (ng/g). *= significant differences [(p <0.05) using one-way ANOVA] in the concentrations of DON and DON-3-G at the same a_w (for each treatment in each row). Sum= Total DON content. <LOD- below limit of detection. <LOQ- below limit of quantitation. EU limit DON: 1250- 1750 µg/kg in unprocessed grains.

Table 4-4: Mean values of concentrations (ng/g) of DON and its bound DON-3-glucoside in irradiated wheat $\pm$ *F. graminearum* (n=4) at different storage conditions.

Irradiated wheat control							Irradiated wheat + <i>F. graminearum</i>				
T (°C)	aw	DON ¹ (ng/g)	DON-3-G ² (ng/g)	3-AcDON ³ (ng/g)	15-AcDON ⁴ (ng/g)	Sum (ng/g)	DON ¹ (ng/g)	DON-3-G ² (ng/g)	3-AcDON ³ (ng/g)	15-AcDON ⁴ (ng/g)	Sum (ng/g)
20	0.93	<LOQ	<LOD	nd	nd	-	<LOQ	<LOD	<LOD	28	28
	0.95	<LOD	<LOQ	<LOD	<LOQ	-	953	36*	<LOD	32	1021
	0.98	<LOQ	<LOQ	<LOQ	<LOD	-	22643	425*	245	261	23575
25	0.93	<LOQ	<LOQ	<LOD	<LOD	-	93	40	<LOD	<LOQ	133
	0.95	<LOQ	<LOD	<LOD	<LOQ	-	2159	35*	<LOD	30	2224
	0.98	<LOQ	<LOD	<LOQ	<LOQ	-	52332	202*	159	188	52881

¹ Deoxynivalenol, ² Deoxynivalenol-3-Glucoside. *= significant differences [(p <0.05) using one-way ANOVA] in the concentrations of DON and DON-3-G at the same a_w (for each treatment in each row). Sum= Total DON content. <LOD- below limit of detection. <LOQ- below limit of quantitation. nd- not detected. EU limit DON: 1250- 1750 µg/kg in unprocessed grains.

4.3.6 Water activity and temperature impact on the concentrations of ZEN and its conjugates in natural wheat grains with and without *Fusarium graminearum* inoculation.

There were no measurable ZEN or ZEN conjugates in the naturally contaminated wheat control samples at 0.93 a_w and 0.95 a_w for both temperatures. Surprisingly, Zearalenone-14-Sulphate (ZEN-14-S) concentrations were more than three times higher than its precursor ZEN at 0.98 a_w at both temperatures. However, temperature rise did not significantly impact its concentrations.

In the naturally contaminated inoculated wheat with *F. graminearum*, ZEN-14-S significantly increased as water activity and temperature increased except at 0.98 a_w at 25 °C. The concentrations of ZEN-14-S alone exceeded ZEN's maximum limit at 0.93 a_w , 20 °C compared to the naturally contaminated wheat where it exceeded the maximum limit at 0.98 a_w , 25 °C.

At all water activities at 20 °C, the concentrations of ZEN-14-S were higher than ZEN, however, at 25 °C, ZEN-14-S had lower concentrations than ZEN for all water activities except at 0.95 a_w . There were no significant differences ($p > 0.05$) in the concentrations of ZEN and ZEN-14-S at all storage conditions except at 0.93 a_w , 20 °C, in the naturally contaminated inoculated wheat (Table 4-5).

4.3.7 Water activity and temperature impact on the concentrations of ZEN and its conjugates in irradiated wheat grains with and without *Fusarium graminearum* inoculation.

In the irradiated wheat control, ZEN was only measurable at 0.98 a_w and 25 °C. Meanwhile, in the inoculated irradiated wheat, ZEN concentrations significantly increased at all storage conditions. At 25 °C, ZEN-14-S concentrations significantly increased with a rise in water activity with concentrations exceeding ZEN's maximum limit. Zearalenone-14-Glucoside (ZEN-14-G) and Zearalenone-16-Glucoside (ZEN-16-G) concentrations were below the limit of detection in both wheat treatments at all storage conditions. Table 4-6 shows the significant differences in the concentrations of free ZEN to all its conjugates at all storage conditions. The impact of each storage condition on the concentrations of ZEN and each of its conjugates and the statistical

differences in the concentration ratios of ZEN to each of its conjugates in all treatments are found in the supplementary materials (Table D-4, Table D-6 - Table D-9).

The interactions that exist among individual storage conditions, including the interaction of a_w x temperature x treatment, have a significant impact on the concentration ratios of ZEN-14-S (Figure D-2 in Appendix D).

4.3.8 Water activity and temperature impact on the concentrations of other secondary metabolites in natural wheat grains with and without *Fusarium graminearum* inoculation.

3-acetyl deoxynivalenol (3-AcDON) concentrations were mostly not significantly different from the concentrations of 15-acetyl deoxynivalenol (15-AcDON) at all storage conditions in all wheat treatments. Their optimum production conditions varied in both natural wheat treatments (Table 4-3) however, their highest concentrations were observed at 0.98 a_w , 20 °C in the inoculated irradiated wheat (Table 4-4).

Nivalenol (NIV) concentrations were highest at 0.95 a_w , 20 °C with mean values of 57.6 ng/g in the naturally contaminated wheat control samples and 71.5 ng/g in the naturally contaminated inoculated wheat at 0.98 a_w , 25°C (data not shown).

Table 4-5: Mean values of concentrations (ng/g) of ZEN and its glucosides in naturally contaminated wheat inoculated with and without *F. graminearum* (n=4) at different storage conditions.

Naturally contaminated wheat control							Natural wheat + <i>F. graminearum</i>				
T (°C)	A _w	ZEN ¹ (ng/g)	ZEN-14-S ² (ng/g)	ZEN-16-G ³ (ng/g)	ZEN-14-G ⁴ (ng/g)	Sum (ng/g)	ZEN ¹ (ng/g)	ZEN-14-S ² (ng/g)	ZEN-16-G ³ (ng/g)	ZEN-14-G ⁴ (ng/g)	Sum (ng/g)
20	0.93	<LOD	<LOD	<LOD	<LOD	-	29.0	107*	<LOD	<LOD	138
	0.95	<LOD	<LOD	<LOD	<LOD	-	620	1527	<LOD	<LOD	2150
	0.98	457	1676	<LOD	<LOD	2133	13401	13790	<LOD	<LOQ	27208
25	0.93	<LOQ	<LOD	<LOD	<LOD	-	386	224	<LOD	<LOD	610
	0.95	<LOD	<LOD	<LOD	<LOD	-	1535	1937	<LOD	<LOD	3477
	0.98	65	442	<LOD	<LOD	507	21973	8899	<LOD	<LOQ	30872

¹ Zearalenone, ² Zearalenone-14-Sulphate, ³ Zearalenone-16-Glucoside, ⁴ Zearalenone-14-Glucoside. *= significant differences [(p <0.05) using one-way ANOVA] in the concentrations of ZEN and ZEN-14-S at the same a_w (for each treatment in each row). Sum= Total ZEN content. <LOD -below Limit of detection. <LOQ- below limit of quantitation. EU limit ZEN: 100 µg/kg in unprocessed grains.

Table 4-6: Mean values of concentrations (ng/g) of ZEN and its glucosides in irradiated wheat inoculated with and without *F. graminearum* (n=4) at different storage conditions.

Irradiated wheat control							Irradiated wheat + <i>F. graminearum</i>				
T (°C)	A _w	ZEN ¹ (ng/g)	ZEN-14-S ² (ng/g)	ZEN-16-G ³ (ng/g)	ZEN-14-G ⁴ (ng/g)	Sum (ng/g)	ZEN ¹ (ng/g)	ZEN-14-S ² (ng/g)	ZEN-16-G ³ (ng/g)	ZEN-14-G ⁴ (ng/g)	Sum (ng/g)
20	0.93	nd	<LOD	<LOD	nd	-	64	nd	<LOD	nd	64
	0.95	nd	nd	nd	nd	-	7851	nd	<LOD	<LOD	7851
	0.98	<LOD	<LOD	<LOD	nd	-	93253	<LOQ	<LOD	<LOD	93281
25	0.93	nd	nd	nd	nd	-	1018	176*	<LOD	<LOD	1195
	0.95	<LOD	<LOD	nd	nd	-	95370	4617*	<LOD	<LOD	99994
	0.98	68	<LOD	<LOD	nd	68	536802	27899*	<LOD	<LOD	564709

¹ Zearalenone, ² Zearalenone-14-S, ³ Zearalenone-16-G, ⁴ Zearalenone-14-G. *= significant differences [(p <0.05) using one-way ANOVA] in the concentrations of ZEN and ZEN-14-S at the same a_w (for each treatment in each row). Sum= Total ZEN content. <LOD -below Limit of detection. <LOQ- below limit of quantitation. nd- not detected. EU limit ZEN: 100 µg/kg in unprocessed grains.

4.4 Discussion

The knowledge of the concentrations of free and conjugated mycotoxins under different environmental storage conditions is not well documented. According to Magan et al. [25], the risk threshold for safe grain storage is below water activity of 0.70 a_w and <15% moisture content (MC). Above these storage conditions, grains are prone to mould spoilage and mycotoxin contamination. This study investigated the impact of storage conditions of water activities 0.93, 0.95, 0.98 a_w (23–28% MC) and temperature (20 and 25 °C) on the concentrations of DON and ZEN to their respective conjugated, DON-3-G and ZEN conjugates. Inadequate drying of grains or storage conditions due to pest infestations, silo leaks, seasonal rainfall, or temperature changes can create these conditions in wheat post-harvest. Naturally contaminated and irradiated wheat grains, either inoculated or not inoculated with *Fusarium graminearum* were investigated. The storage conditions were chosen as they represent the marginal conditions for *Fusarium graminearum* growth and the production of DON and other trichothecenes toxins [29]. Generally, mycotoxin concentrations were higher in the inoculated irradiated wheat grains than in the naturally contaminated treated wheat grains.

Impact of water availability and temperature on the concentrations of DON and DON-3-G.

In the naturally contaminated wheat control samples, DON-3-G had its highest concentrations of co-occurrence with DON at 0.93 a_w at 25 °C with a proportion ratio of 56%: 44% where DON levels were least observed. This trend was similar to the initial concentrations of DON-3-G: DON at a drier condition (0.67 a_w) in the wheat before the storage experiment. While other reasons for higher concentrations of DON glucoside at the drier conditions may not be fully understood in this study, we know that *Fusarium graminearum* thrives more in a very wet condition which may subsequently lead to higher production of DON than DON-3-G. High concentrations of DON-3-G compared with DON were found

in beer and brewing intermediate products from barley with the highest concentrations of DON-3-G at 37 µg/l compared to DON's highest concentrations of 23 µg/l [36]. In contrast to our findings, several studies reported DON-3-G concentrations lower than DON levels in cereal grains and cereal-based products [19,37,38]. Sasanya et al. [39] documented the average concentrations of DON-3-G (0.2 µg/g) to be lower than average concentrations of DON levels (1.4 µg/g) in red hard spring wheat. An explanation for this contrast could be attributed to the variety of grains or harvest seasons. In addition, these studies did not investigate the co-occurrence of DON and its conjugates under different water and temperature conditions as used in our study, thus making direct comparisons difficult.

Other studies have reported an increase in DON concentrations by its conjugates [20,40,41]. Streit et al. [23] reported that DON-3-G levels could increase the total DON content in contaminated feeds. Their findings revealed that DON-3-G with a maximum value of 7764 µg/kg contributed an average of 12% of total DON content (maximum value 25,928 µg/kg) in samples analysed. Although DON was present in all the 89 samples analysed, DON-3-G was present in 69 out of 89 samples.

Interestingly, a reciprocity effect was observed, as DON-3-G levels increased with temperature rise, DON levels decreased. However, a rise in the water activity levels had a significant impact on DON production at both temperatures (Table 4-3). The pattern of DON concentrations increasing with water activity but decreasing with temperature was supported by Garcia-Cela et al. [42]. They reported the production of type B trichothecenes in two wheat grain treatments stored under different temperatures and a_w conditions. At the wettest condition examined (0.95 a_w) for both natural wheat treatments, the concentrations of DON decreased with temperature rise from 20 to 25 °C. Their result agrees with the findings of DON in our study. As the population of mycotoxigenic *Fusarium graminearum* increased in the naturally contaminated wheat through inoculation, the concentrations of DON increased significantly than DON-3-G concentrations at all storage conditions.

Similarly, in the inoculated irradiated wheat samples in Table 4-4, the concentrations of DON increased with a rise in water activity and were highest at 0.98 a_w and 25 °C. This result is in line with those from Comerio et al. [43] who reported optimal production of DON at 0.98 a_w at the only temperature studied, 25 °C. In contrast to our results, Ramirez et al. [26] reported DON levels to be maximum at 30 °C but rapidly produced at 25 °C. However, several differences were noted in the study. Firstly, the water activity level used was 0.995 a_w . Two Argentinian *Fusarium graminearum* strains were inoculated in irradiated wheat grains, and incubated for 42 days, rather than the 18 days used in this study. The range of DON production for both strains was 135,456 and 98,446 ng/g which were higher than DON levels in the inoculated irradiated wheat in our study. The high DON levels may be attributed to strain types and longer storage days under very wet conditions.

Table 4-3 also shows that the concentrations of 3-acetyl deoxynivalenol (3-AcDON) at all storage conditions were not significantly different from 15-acetyl deoxynivalenol (15-AcDON) concentrations in the natural wheat treatments. This trend was observed by Streit et al. [23] who reported 15-AcDON levels (2718 µg/kg) higher than 3-AcDON levels (588 µg/kg) in the same maize cob samples examined.

Impact of water availability and temperature on the concentrations of ZEN and its conjugates.

ZEN-14-S dominated most wheat treatments and had the highest concentrations among all the conjugates of zearalenone analysed. ZEN-14-S concentrations at 0.98 a_w and both temperatures in the naturally contaminated wheat control samples raise a major concern for food safety as its concentrations exceeded the maximum limit set for ZEN (Table 4-5). A similar trend was observed in the naturally contaminated wheat inoculated wheat with *Fusarium graminearum*, ZEN-14-S concentrations exceeded ZEN's maximum limit (100 µg/kg) at all storage conditions analysed. It was interesting to note that there were no significant differences in the concentrations of conjugated ZEN-14-S and ZEN at

all storage conditions except at 0.93 a_w and 20 °C. There were no measurable ZEN-14-G and ZEN-16-G concentrations in the natural and irradiated wheat treatments at all storage conditions.

Mylona et al. [44] showed that the relationship between water activity and temperature significantly affects ZEN production in wheat grains infested with *Fusarium* species. Similar to our findings, the highest concentrations of ZEN were observed at 0.97 a_w and 25°C in the irradiated wheat grains. Our study indicated the highest concentrations of ZEN occurred at 0.98 a_w and 25°C in the inoculated irradiated wheat treatment. Another study with irradiated wheat reported ZEN and its metabolites (alpha-ZOL and beta-ZOL) production to be optimal at 0.90 a_w , 25 °C in irradiated wheat while optimal production condition in naturally contaminated wheat was 0.93 a_w , 25 °C [45]. These studies have suggested that storage conditions for natural grains such as > 0.93 a_w and >20 °C promote the risk of ZEN production while our study suggested that under similar conditions, there is a potential risk of the production of ZEN conjugates.

Analytically, ZEN-14-S is regarded as an unstable analyte [46]. Our findings showed higher concentrations of ZEN-14-S in the naturally contaminated inoculated wheat than in the irradiated inoculated wheat at 20 °C, one could attribute the increase to other fungal genera in the microbial profile in the naturally inoculated wheat. It was reported that strains of *Aspergillus* and *Rhizopus* can bio-transform ZEN to its sulphate and glucoside forms [47–49].

Impact of water availability and temperature on the concentrations of emerging mycotoxins

Apart from the commonly regulated mycotoxins, another metabolite found in both natural wheat treatments is moniliformin ranging from 43–5492 ng/g which increased with a rise in both a_w and temperature. The highest concentration of moniliformin was at 0.95 a_w and 25 °C. Concentrations were low in the inoculated irradiated samples regardless of *F. graminearum* inoculation (data not shown).

Although present at low concentrations and mostly below the limit of detection (LOD), diacetoxyscirpenol (DAS) and enniatins (A, A1, B, and B1) were found in the wheat treatments. The optimum condition for enniatins production was 0.98 a_w at 25 °C in both naturally contaminated wheat treatments while no measurable concentrations occurred at all storage conditions for the irradiated wheat treatments. This agrees with a previous study reporting 0.95 a_w , 20–25 °C as the optimal conditions for enniatins production, although 0.95 a_w was the highest water activity used in the study [42].

In general, our results highlighted the *Fusarium* toxins (free, conjugated, and emerging) produced in naturally contaminated wheat stored under high water stress conditions. A summary of these *Fusarium* toxins found is shown in Figure 4-3, adapted from Magan et al. [25].

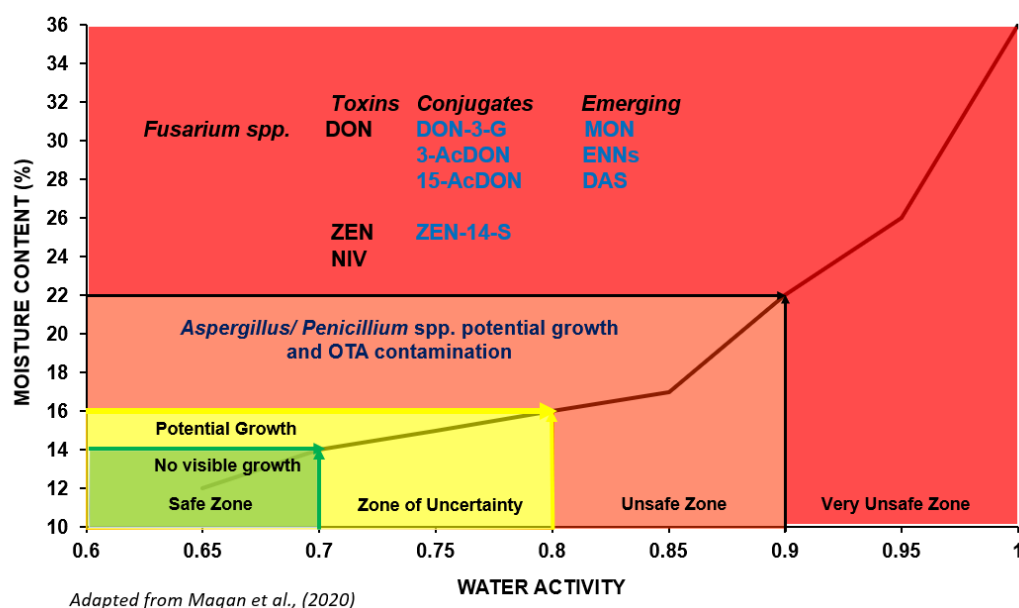


Figure 4-3: The effect of water availability on the relative contamination with free, masked, and emerging mycotoxins in wheat. DON: Deoxynivalenol, DON-3-G: Deoxynivalenol-3-glucoside, AcDON: Acetyl-deoxynivalenol, ZEN: Zearalenone, ZEN-14-S: Zearalenone-14-Sulphate, NIV: Nivalenol, DAS: Diacetoxyscirpenol, ENNs-Enniatins (A, A1, B, B1), OTA- Ochratoxin A. Toxins in black print: already described/regulated toxin. Toxins in blue print: potential toxins at storage conditions described in this research.

4.5 Conclusion

This study shows that the ratios of DON and ZEN to their bound mycotoxin concentrations varied under different environmental conditions. Although DON levels in the naturally contaminated wheat were below the EU maximum limit, the total DON content value in the contaminated wheat is underestimated if the concentration values of the conjugated glucoside and acetylated forms of DON are not considered. DON-3-G and ZEN-14-S concentrations were highest at 0.98 a_w at both temperatures and treatments. In general, water activity significantly impacted the concentrations of each analyte while temperature change did not significantly impact on the concentrations of each analyte. However, the question remains of how high temperature and high-water activity influence grain enzymes to bio-transform parent toxins to their conjugated forms in wheat despite irradiation treatment. The increase in conjugated mycotoxin levels underlines the importance of efficient drying post-harvest and effective monitoring of grains in storage. This study stressed the importance of the knowledge of the storage conditions for both free and conjugated mycotoxin production for risk assessment for food safety. Furthermore, the concentrations of conjugated mycotoxins could potentially increase the total mycotoxin content in contaminated cereal grains, therefore robust analysis should be engaged during trading or export checks, to avoid underestimation of mycotoxin content and ensure food and feed grains are safe for consumption.

4.6 References

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5 The effect of the interactions of water activity, and temperature on OTA, OTB, and OT α produced by *Penicillium verrucosum* in a mini silo of natural and inoculated wheat using CO₂ production as fungal activity sentinel.

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Abstract

Ochratoxin A (OTA) is a nephrotoxin that contaminates grains in storage. Moisture and temperature sensors give delayed responses due to their slow kinetic movement within the silo. This study examines if CO₂ production could predict OTA contamination and identify storage conditions exceeding the maximum limit (5 µg/kg). The impact of water activity levels (0.70–0.90 *a_w*), temperatures (15 and 20 °C), and storage duration on (a) *Penicillium verrucosum* population, (b) CO₂ respiration rates (RR), and (c) ochratoxins concentrations in stored wheat was investigated. 96 samples were analysed for ochratoxins with LC-MS/MS. RR was >7 times higher at wetter conditions than at drier *a_w* levels. A positive correlation between CO₂, OTA, OTB, and OTα was observed at the wettest conditions. OTA exceeded the limit at ≥ 0.80 *a_w* (16% moisture content) with RR ≥ 15 µg CO₂ kg⁻¹ h⁻¹. The knowledge of the RR of stored grain would alert grain farmers/managers to improve grain storage management.

Keywords: *Penicillium verrucosum*, ochratoxin A, carbon dioxide, respiration rate, water activity, LCMS/MS.

5.1 Introduction

Penicillium verrucosum is a competitive fungal species dominating cereal grains pre- and post-harvest under conducive environmental conditions [1]. Its occurrence in grains indicates ochratoxin A contamination and is prevalent in temperate regions with high humidity such as Northern Europe [2–4]. OTA is also produced by *Aspergillus ochraceus*, *Aspergillus carbonarius*, and sometimes *A. niger* mostly under warmer climatic conditions [5–8]. OTA has been reported to occur in a wide variety of foods ranging from cereal grains, cereal products, cheese and meat products, dried fish, nuts, beans, pepper, grapes, wine, and dried fruits [9–11]. OTA is a potent nephrotoxin, carcinogen, and immunosuppressant, posing significant health risks to humans and animals upon ingestion [12,13]. Chronic exposure to OTA through contaminated wheat products has been associated with various adverse health effects, including renal diseases, immune system disorders, and urinary tract tumours [14]. The International Agency for Research on Cancer (IARC) classifies OTA as a possible human carcinogen (Group 2B) based on sufficient evidence of its carcinogenicity in animals and limited evidence in humans [15].

OTA, within the family of structurally related ochratoxins, stands out as the most potent toxin. Its toxicity surpasses that of its dechlorinated counterpart, Ochratoxin B (OTB), yet remains similar to the toxic effects observed with its ethyl esterification derivative, Ochratoxin C (OTC) [16,17]. Both OTB and OTC have been implicated in various adverse health effects, including immunotoxicity, hepatotoxicity, kidney toxicity, genotoxicity, and cytotoxicity [18–20]. Although OTB has been detected in a range of food items such as cereals, coffee, and wine, its presence generally occurs at lower levels compared to ochratoxin A.

Ochratoxin alpha (OT α), a less potent variant of OTA, emerges through chemical alterations in OTA's structure during metabolism or food processing [20–22]. Abrunhosa et al. [23] elucidated the enzymatic hydrolysis of OTA's peptide bond, catalyzed by numerous peptidases and lipases, leading to the formation of OT α . Additionally, Ringot et al. [24] documented OT α 's generation from OTA metabolism by rumen microorganisms following ingestion. Consequently, OT α

has been identified as a reliable biomarker for OTA in various biological matrices, including raw milk, plasma, and urine in both humans and animals [25–27]. A maximum limit of 5 µg/kg has been imposed by the European Commission for OTA in cereals and cereal products. The process of determining a maximum limit for OTC, OTB, and OTα is still in progress. Consequently, it is imperative to focus on detecting and minimising these compounds in food and animal feed.

The growth of *Penicillium verrucosum* and OTA production are influenced mostly by the moisture content of grains and temperature within the storage environment and storage practices [1]. OTA reduces the quality and safety of stored grains resulting in significant economic losses for farmers and traders and poses significant health risks when consumed [28,29]. Cairns-Fuller et al. [30] examined the growth rate and OTA production by *P. verrucosum* under different conditions of a_w , temperature, and gas compositions using wheat grains and a 2% wheat-based medium. Optimum growth was established *in vitro* on the wheat-based media at 0.98 a_w at 25 °C and *in vivo* in wheat grains at 0.95 a_w at 25°C. The report also shows minimal growth at 0.85 a_w with no production of OTA after 56 days of incubation.

Other studies reported that higher water availability > 0.95 a_w and temperatures > 28 °C could impact the metabolism of *P. verrucosum* for low growth rate and OTA formation in cereal grains and cereal products [31–34]. This suggests that OTA synthesis can occur within a certain range of water activity levels smaller than the levels for the growth of *P. verrucosum* which can still thrive at low water activity levels. The detection of OTA produced by *Aspergillus* species in different foods *in vitro* and *in situ* has been largely investigated [35–37]. Only a few studies have considered OTA produced by *Penicillium verrucosum* species. Also, studies on the effects of the interactions of environmental conditions on the growth of *P. verrucosum* and OTA, OTB, and OTα production in stored wheat are limited.

In addition to temperature and water availability that influence grain deterioration in storage, it is important to consider the gaseous state of the storage environment. Post-harvest grains continue to respire during storage with the results of the metabolic process as CO₂, heat, and water [38]. In addition, the

presence of living organisms such as fungi and insects may increase the CO₂ levels within the storage environment [39]. Therefore, monitoring the CO₂ concentrations released in grain silos could indicate the onset of spoilage and this can be measured using CO₂ sensors. Due to the rapid result alertness from the CO₂ sensors, it is preferable to moisture and temperature sensors to monitor grain quality in storage [40–42].

Several studies have used CO₂ measurement to monitor grain quality in storage. Mylona et al. [43] used the cumulative CO₂ production data measured with Gas Chromatography to quantify the effect of different storage conditions (a_w : 0.89 – 0.97) and temperature (15– 30°C) on dry matter loss relating it to deoxynivalenol, zearalenone, and fumonisins in stored wheat and maize. Their findings indicated that CO₂ production increased as a_w and temperature increased, showing distinct production patterns for the three toxins. A similar method and results were reported by Garcia-Cela et al. [44]. However, these studies did not measure the real-time respiration rates in the stored grains, and the relationship between the CO₂ produced, toxins produced, and the grain mycobiota in storage was not established. Therefore, to accurately evaluate grain storage conditions using CO₂ level measurements, it's crucial to understand the CO₂ production rate of wheat and its dynamics at different moisture levels and temperatures.

This study aimed to examine if real-time CO₂ production in stored wheat silos could predict the early risk of OTA contamination and identify storage conditions exceeding maximum limit (5 µg/kg). The objectives of this study are to investigate the impact of storage conditions (0.70 - 0.90 a_w , 15 and 20 °C) on (a) *Penicillium verrucosum* populations (b) CO₂ respiration rates (RR) (c) ochratoxins concentrations produced in naturally contaminated and *Penicillium verrucosum* inoculated wheat and (d) to examine the relationship between the CO₂ and ochratoxins concentrations in the contaminated wheat.

5.2 Materials and Methodology

5.2.1 Wheat grains and moisture adsorption curve

Wheat grains from Northern Ireland (2021 harvest) were used in the study. They were stored 6 months before the storage experiment. The initial water activity of grains was analysed using (AquaLab water activity meter 4 TE Decagon devices, Inc, Pullman, USA). For the moisture content analysis, grains were oven-dried at 105 °C overnight and were calculated on a dry weight basis.

10 g sub-samples of wheat were placed in ten 25 mL glass Universal bottles. Known amounts of water (0.1–3.5 mL) were added to the grains. The bottles were sealed and mixed thoroughly and kept at 4 °C for 24 hours with regular shaking. The samples were equilibrated at room temperature for 1 hour and the water activity of grains was measured (AquaLab water activity meter 4 TE Decagon devices, Inc, Pullman, USA). Moisture contents (MC) of the samples were calculated following oven drying at 105 °C overnight. Three replicates were analysed. The amount of water added was plotted against a_w values to achieve the targeted water activity levels for grain modification. The relationship between the MC (dry weight basis) and a_w values was plotted and noted.

5.2.2 Fungal strain and spores harvesting

Penicillium verrucosum (OTA11) known to produce ochratoxins was used in this study. The strain was stored at –20 °C in glycerol: water (70:30, v/v) in the Magan Centre of Applied Mycology culture collection, Cranfield University. *Penicillium verrucosum* was grown on Malt Extract Agar (MEA) with an anti-bacterial agent (chloramphenicol) incubated at 25 °C for 7 days.

Spores were harvested aseptically from the 7-day-old *Penicillium* plates scraped with a sterile spatula with sterile Tween 80 water (0.05% v/v). 10mL of the suspension was transferred into a sterile tube and shaken. Spores targeted concentrations (1.5×10^5 spores/mL) were counted and calculated with the Thoma cells formula using an Olympus microscope (U-ULH model, Olympus

Optical, Japan). The spore suspension was further diluted with Tween 80 water to achieve the targeted volume of 20 mL for the inoculation experiment.

5.2.3 Mini-silo CO₂ sensing storage experiment.

3 kg of wheat grains were weighed into a 12-Litre box. The grains were rehydrated to targeted water activities (0.70, 0.80, 0.90, 0.95 *a_w*) with a known amount of water and 20 mL less for spore inoculum for inoculated grains. The grains were vigorously mixed and stored at 4 °C overnight. The following day, the grains were equilibrated at the targeted temperature (15 and 20°C) rooms for 3 hours and the water activities of the equilibrated grains were measured. Grains were transferred into a 5-litre coffee flask with a centrally placed ATEX-compliant sensor probe. Cotton wool was placed in the headspace of the flask to minimise gaseous movement in and out of the flask. There were two flasks (replicates) for each water activity level. The ten flasks were stored at each temperature condition. The probe comprises CO₂, temperature, and relative humidity sensors connected to a computer system where their respective real-time monitoring readings from the stored grains were collected. The grains were stored for 20 days. 60g of contaminated samples were collected on day 10 and day 20 from each flask with a sterile probe into sterile jars having a good representation of the bulk sample. The water activity of the grains was monitored before and after sampling. 40 g of the sub-sample grains were dried in the oven at 50–55 °C overnight, ground, and stored at –20 °C before further analysis. The CO₂ sensors were calibrated before each experiment for each treatment studied. After sampling on day 10, the grain inside the flask is 60 g less, the new weight is considered for calculating the respiration rates for day 20. The respiration rate was calculated using the diffusion formula in Equation 5-1 adapted from Raudienė et al. [42].

$$\text{Respiration rates} = \frac{\Delta\text{CO}_2 \times \text{MCO}_2 \times \text{Vh}}{\text{Vm} \times \text{m} \times \Delta\text{t}} \quad (5-1)$$

where ΔCO_2 is the change in CO_2 volumetric concentration in ppm, M_{CO_2} is the molar mass of CO_2 gas = 44.01 (g mol^{-1}), V_h is the volume of headspace in the jar (L), V_m is the molar volume of a gas (L mol^{-1}), m is the mass of the wheat sample (kg) and Δt is the duration (h) of ΔCO_2 .

The molar volume was determined by analyzing the storage temperatures.

$$V_m = \frac{R \times T}{P} \quad (5-2)$$

where R is the gas constant ($\text{L mol}^{-1} \text{K}^{-1}$), T is temperature (K), P is pressure (atm)

5.2.4 Fungal populations and isolations

5.2.4.1 Fungi Enumeration (serial dilution method)

1g of whole kernel contaminated grain was weighed and soaked in sterile 9 mL peptone water in a 25 mL universal bottle for 3 hours. 1 mL was transferred into the 10^{-2} dilution bottle and was serially diluted to the 10^{-4} bottle. Sterile tips were used between each dilution and the universal bottle's content was shaken using a vortex. 100 μL of the dilution aliquots were reversely plated onto Dichloran Glycerol 18 agar (DG18+: Oxoid CM0729) and Malt Extract Agar (MEA+: Oxoid CM59) media in 3 replicates and spread out with a sterile metal spreader. The plates were incubated at 25 °C for 7 days. Fungal genera were identified visually with a stereoscope. Colonies were counted with a colony counter and expressed as $\log_{10}$ CFUs/g calculated on a dry weight basis.

5.2.4.2 Fungi isolation (direct plating method)

25 grains were directly inoculated on DG18+ and MEA + media to identify dominant fungal genera in the contaminated grains. 5 grains were placed equidistant on each medium plate of five replicates and incubated at 25 °C for 7

days. Fungal genera were identified visually with a stereoscope and morphological characteristics. The frequency of isolation was calculated using Equation 5-3 below.

$$\% \text{ Frequency of isolation} = \frac{\text{Total contaminated grains}}{\text{Total grains in plates (25 grains)}} \times 100 \quad (5-3)$$

5.2.5 Mycotoxin analysis

The initial mycotoxin concentrations of the wheat grains were analysed. After the storage experiment, 96 contaminated wheat samples (including replicates) were analysed for mycotoxins. 5 g of ground contaminated wheat grains were mixed with 20 mL of the extraction solvent (acetonitrile (ACN): water (H₂O): acetic acid (HAc) 79: 20:1, v/v/v). Samples were extracted for 90 minutes using a GFL Orbital Shaker 3017 (Berlin, Germany). After extraction, 500 µL of the extracted aliquot was diluted with 500 µL dilution solvent (ACN: H₂O: HOAc 20:79:1, v/v/v), yielding a 1:1 dilution in a vial. The mix was vortexed and 5 µL of the diluted extracts were injected into the Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) system. The chromatographic method and mass spectrometric parameters were adapted from Sulyok et al. [45]. The accuracy of the method is verified on a continuous basis by participation in a proficiency testing scheme organised by BIPEA (Gennevilliers, France). The rate of satisfactory z-scores of $-2 < z < 2$ is $> 96\%$ for the 2200 results submitted so far. The limit of detection (LOD) and quantification (LOQ) of OTA and OTα are 0.08 µg/kg and 0.28 µg/kg respectively while for OTB, LOD was 0.12 µg/kg and LOQ was 0.39 µg/kg.

5.2.6 Statistical analyses

The data sets were analysed using Statistica 14.0.1, JMP® Pro 17, Microsoft Excel, and RStudio in R 4.4.1. The normal distribution of data was examined with the normal plots of the residuals. When data failed the normality test, data were transformed to square root or logarithm values to achieve normality. Transformed data were normally distributed; therefore, factorial ANOVA was used to analyse the effect of the interactions between the storage conditions on respiration rate and ochratoxin concentrations. The Tukey Honest Significant Difference (HSD) post hoc test was used to evaluate differences among means for the interactions of storage conditions. The non-parametric comparison for each pair (Wilcoxon method), evaluates significant differences in fungal populations and mycotoxin concentrations for each storage condition. Statistical analyses performed were considered significant when p values were <0.05 . The concentrations of ochratoxins below the limit of detection and quantitation were assigned with values of LOD/2 and LOQ/2, respectively, for statistical analysis [46]. A linear regression model was done in R Studio software on transformed data to examine the relationship between CO₂, and OTA concentrations in both wheat treatments.

5.3 Results

5.3.1 Visual images of contaminated grains.

The visual representation of *Penicillium* contamination in wheat grains is illustrated in Figure 5-1.

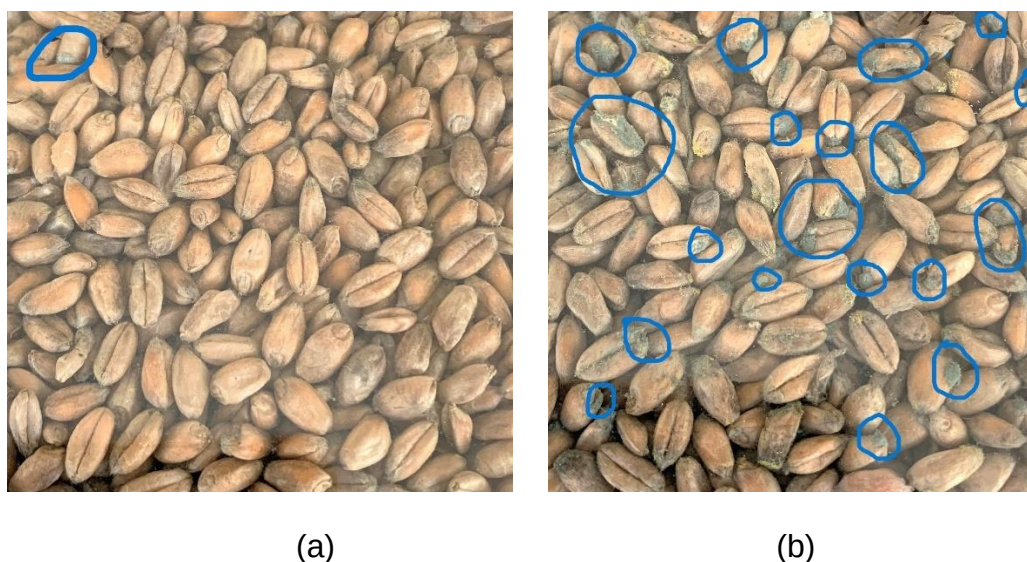


Figure 5-1: Images showing contaminated wheat grains on day 20 at 15 °C with *Penicillium* growth at (a) 0.90 a_w and (b) 0.95 a_w . Grains at drier levels, 0.70 and 0.80 a_w , showed no visible mould growth.

5.3.2 Fungal populations in contaminated grains

Table 5-1 shows the mean values of the colony-forming units of *Penicillium* populations in both wheat treatments and media. In the naturally contaminated wheat at 15 °C, *Penicillium* populations were high at 0.95 a_w but significantly decreased with temperature rise. Meanwhile, at 20 °C, *Penicillium* populations were highest at 0.90 a_w . In the inoculated wheat, *Penicillium* populations were highest at 0.90 a_w at both temperatures. A similar trend in population pattern was observed in both wheat at 20 °C.

in Table

In Table 5-1 below, the nonparametric comparison for each pair using the Wilcoxon method shows significant differences in the fungal populations for 0.90 and 0.95 a_w on MEA + media on day 10 and 15 °C for both NW and PVW treatments. For DG18+ media, significant differences were observed for 0.90 and 0.95 a_w on day 10 and 15 °C for NW and day 20 and 15 °C for NW.

The effect of the interactions of the storage conditions and their p values on *Penicillium* populations are shown in Table 5-2 while the mean values of the frequency of isolation of *Penicillium* in both wheat treatments and media are in Table 5-3.

Table 5-1: Mean values ($n= 6 \pm SE$) of *Penicillium verrucosum* populations ($\log_{10}$ CFUs/G) in contaminated grains at all storage conditions.

Grains	T (°C)	Days	0.70 a_w		0.80 a_w		0.90 a_w		0.95 a_w	
			MEA+	DG18+	MEA+	DG18+	MEA+	DG18+	MEA+	DG18+
NW	15	10	ng	ng	ng	2.6 ± 0.0	4.3 ± 4.1	3.8 ± 3.6	6.9 ± 6.5	6.9 ± 6.4
		20	ng	ng	ng	ng	6.4 ± 4.9	6.2 ± 5.7	4.5 ± 4.4	4.5 ± 4.4
	20	10	ng	2.6 ± 2.5	3.3 ± 3.2	5.2 ± 5.0	5.4 ± 4.9	5.5 ± 5.4	2.3 ± 2.6	ng
		20	2.8 ± 2.3	3.1 ± 3.1	5.4 ± 5.1	5.0 ± 4.9	6.4 ± 4.9	6.3 ± 5.8	4.6 ± 4.6	4.6 ± 4.6
PVW	15	10	3.3 ± 3.2	ng	4.6 ± 3.3	4.9 ± 4.2	7.0 ± 0.0	7.0 ± 5.3	6.9 ± 6.2	7.0 ± 6.4
		20	ng	ng	5.5 ± 5.4	5.3 ± 5.2	7.4 ± 6.8	7.3 ± 6.0	7.4 ± 7.3	7.2 ± 7.1
	20	10	3.1 ± 2.6	2.9 ± 0.0	4.5 ± 4.4	4.6 ± 4.4	6.9 ± 6.3	6.0 ± 5.3	6.1 ± 6.0	6.2 ± 6.1
		20	3.0 ± 2.3	2.3 ± 2.3	5.3 ± 4.7	5.2 ± 4.1	7.0 ± 6.9	7.1 ± 6.9	7.1 ± 6.9	7.2 ± 7.1

S.E: Standard error. NW: Naturally contaminated wheat. PVW: *Penicillium verrucosum* wheat. T: Temperature. a_w : water activity. DG18+: Dichloran Glycerol 18 agar + Chloramphenicol. MEA+: Malt Extract Agar + Chloramphenicol. ng: no growth Nonparametric comparison for each pair using the Wilcoxon method mostly shows no significant differences in the fungal populations among the three a_w levels for each media for each day and each temperature (within each row).

Chapter 5

Table 5-2: The effect of the interactions of storage conditions and their p values on *Penicillium* populations in contaminated wheat

Interaction effect	P values
Treatment	0.000000
a_w	0.000000
Temp	0.394802
Days	0.039937
Treatment*a_w	0.000000
Treatment*Temp	0.108158
a_w*Temp	0.000000
Treatment*Days	0.576133
a_w*Days	0.000260
Temp*Days	0.000010
Treatment*a_w*Temp	0.002929
Treatment*a_w*Days	0.000000
Treatment*Temp*Days	0.033348
a_w*Temp*Days	0.000001
Treatment*a_w*Temp*Days	0.033381

a_w-water activity. Temp- temperature

Table 5-3: Frequency of isolation (%) of *Penicillium verrucosum* in contaminated grains at all storage conditions.

Grains	T (°C)	Days	0.70 a_w		0.80 a_w		0.90 a_w		0.95 a_w	
			MEA+	DG18+	MEA+	DG18+	MEA+	DG18+	MEA+	DG18+
NW	15	10	8	6	10	6	98	100	88	100
		20	2	8	10	18	100	100	ng	34
	20	10	6	18	18	24	100	100	22	ng
		20	22	22	98	62	100	100	34	48
PVW	15	10	100	82	100	100	100	100	100	100
		20	100	100	100	100	100	100	78	100
	20	10	96	98	100	100	96	98	ng	50
		20	100	63	100	100	58	62	26	46

NW: Naturally contaminated wheat. PVW: *Penicillium verrucosum* wheat. T: Temperature. a_w : water activity. DG18+: Dichloran Glycerol 18 agar + Chloramphenicol. MEA+: Malt Extract Agar + Chloramphenicol. ng: no growth.

5.3.3 CO₂ production in contaminated wheat.

5.3.3.1 Respiration rates in wheat.

The respiration rates (RR) were steady for the first 4 days in the naturally contaminated wheat at 0.90 a_w while in the inoculated wheat, an increase in RR was observed after day 1 at both temperatures (Figures 5-3). As the temperature and water activity level of grains increased, RR increased. RR at 0.90 a_w significantly differed between both treatments throughout the storage experiment. RR at 0.95 a_w was significantly different in both treatments for the first 8 days at 15 °C and the first 3 days at 20 °C. The mean values of respiration rates ($\mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$) in contaminated wheat at all storage conditions are shown in Table 5-3 while its graphical representation is shown in Figure E-1 in Appendix E.

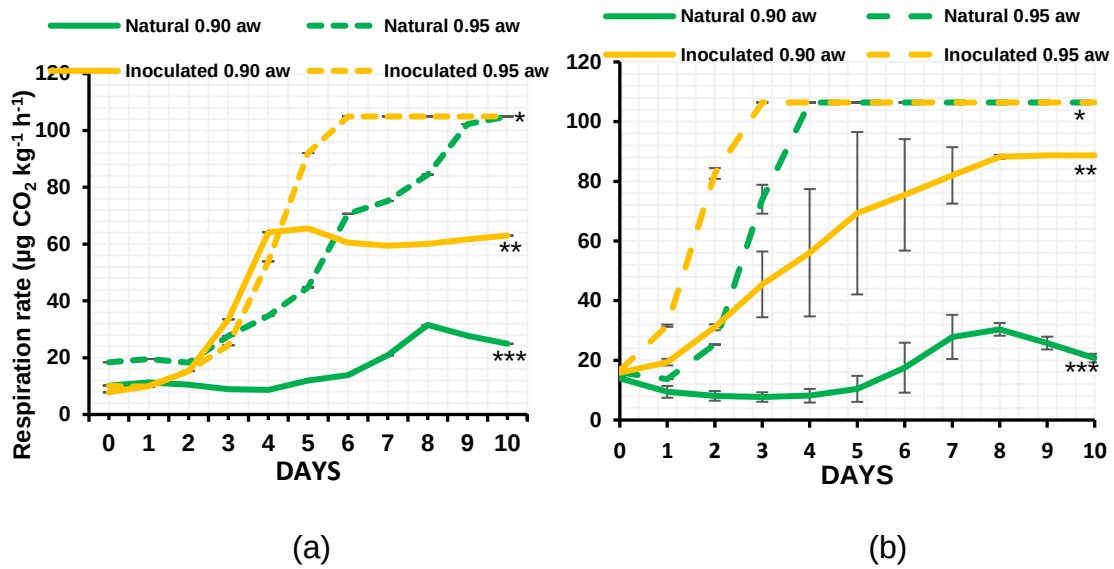


Figure 5-2: The respiration rates in contaminated wheat for the first 10 days at 0.90–0.95 a_w for both treatments at (a) 15 °C and (b) 20 °C with error bars (n=2). a_w - water activity. Natural- naturally contaminated wheat. Inoculated- *P. verrucosum* inoculated wheat. (n=2). a_w - water

Table 5-4: Mean values (n=2) of respiration rates ($\mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$) in contaminated wheat.

Grains	T (°C)	Days	0.70 a_w	0.80 a_w	0.90 a_w	0.95 a_w
Naturally contaminated wheat	15	0	0.9 ± 0.3^a	0.7 ± 0.1^a	5.5 ± 1.7^b	14.6 ± 4.8^c
		10	1.6 ± 0.4^a	1.2 ± 0.1^a	13.5 ± 2.6^b	106.7 ± 4.8^c
		20	1.6 ± 0.4^a	1.3 ± 0.2^a	13.6 ± 4.1^b	106.7 ± 1.7^c
	20	0	2.2 ± 0.6^a	4.9 ± 0.1^a	14.0 ± 0.4^b	15.9 ± 0.9^b
		10	1.2 ± 0.7^a	8.3 ± 0.8^b	20.8 ± 1.0^c	106.4 ± 0.0^d
		20	1.8 ± 0.6^a	8.6 ± 0.7^b	16.1 ± 1.2^b	111.4 ± 0.2^c
<i>Penicillium verrucosum</i> inoculated wheat	15	0	2.0 ± 0.7^a	3.9 ± 0.1^a	7.8 ± 1.7^a	1.8 ± 1.3^a
		10	8.0 ± 2.2^a	22.4 ± 4.1^b	63.0 ± 16.9^c	108.4 ± 0.0^d
		20	8.9 ± 0.3^a	13.9 ± 1.7^a	84.1 ± 1.9^b	108.4 ± 0.0^c
	20	0	3.1 ± 0.7^a	5.3 ± 0.2^a	15.9 ± 2.0^b	16.7 ± 0.9^b
		10	2.4 ± 0.8^a	13.3 ± 0.3^b	88.7 ± 0.0^c	106.4 ± 0.0^c
		20	4.8 ± 1.3^a	17.5 ± 1.7^b	91.4 ± 0.1^c	111.1 ± 0.1^c

S.D- Standard deviation. T- Temperature. a_w - Water activity. Using the Tukey HSD test, different letters show significant differences in the RR for each a_w and days in each row.

5.3.4 The influence of storage conditions on respiration rates in contaminated wheat

Respiration rates significantly increased with the rise in water activity and differed substantially between wheat treatments. Respiration rates were more than seven times higher at wetter conditions than at drier a_w levels. The interactions between a_w x temperature, a_w x treatment, a_w x temperature x treatment, and a_w x days x treatment significantly affected the respiration rates in both wheat treatments (Table 5-4).

5.3.5 The influence of storage conditions on ochratoxin production in contaminated wheat.

The mean values of all ochratoxin concentrations in the contaminated wheat at all storage conditions are in Table 5-4.

Table 5-5: Mean values (n=2 ± S.D) of ochratoxins in contaminated wheat.

Treatment	T (°C)	a _w	OTA (µg/kg)		OTB (µg/kg)		OTα (µg/kg)	
			DAY 10	DAY 20	DAY 10	DAY 20	DAY 10	DAY 20
Naturally contaminated wheat	15	0.7	<	< LOD	<	< LOD	<	< LOD
			LOD		LOD		LOD	
		0.8	<	< LOD	<	< LOD	<	< LOD
			LOD		LOD		LOD	
		0.9	<	17 ± 16.6	<	5 ± 5.7	<	< LOD
			LOD		LOD		LOD	
	20	0.95	873 ± 59.7	1593 ± 535.8*	60 ± 30.3	61 ± 21.0	<	61 ± 1.54
							LOD	
		0.7	<	< LOD	<	< LOD	<	< LOD
			LOD		LOD		LOD	
<i>Penicillium verrucosum</i> inoculated wheat	15	0.8	<	< LOD	<	< LOD	<	< LOD
			LOD		LOD		LOD	
		0.9	<	6 ± 3.6	<	1 ± 0.2	0.4 ± 0.0	< LOD
			LOQ		LOD		LOD	
		0.95	47 ± 8.9	25 ± 9.7	4 ± 3.6	4 ± 0.3	8 ± 3.6	7 ± 5.1
	20	0.7	<	< LOD	<	< LOD	<	< LOD
			LOD		LOD		LOD	
		0.8	<	< LOD	<	< LOD	<	< LOD
			LOD		LOQ		LOD	
		0.9	7 ± 5.8	331 ± 280*	0.8 ± 0.9	16 ± 18.9*	<	< LOD
							LOD	
		0.95	<	54 ± 47.7*	<	3 ± 3.2	<	< LOD
			LOD		LOD		LOD	
		0.7	<	< LOD	<	< LOD	<	< LOD
			LOD		LOD		LOD	
		0.8	<	< LOD	<	1.3 ± 1.1	<	< LOD
			LOD		LOD		LOD	
		0.9	181 ± 147.9	1091 ± 953.4*	36 ± 30.4	126 ± 92.4*	0.6 ± 0.6	2 ± 1.4
		0.95	10 ± 7.7	7 ± 4.2	<	0.4 ± 0.4	<	2 ± 0.4
					LOQ		LOD	

OTA-Ochratoxin A. a_w- water activity. T- Temperature. * represents significant differences (Tukey HSD test) in ochratoxins concentrations between days for each treatment in each row.

5.3.5.1 Ochratoxin A

The concentrations of OTA in the wheat were significantly affected by water activity and storage days. Various interactions significantly influenced OTA

concentrations except for the interactions between days x treatment, temperature x days x treatment, and a_w x temperature x days x treatment. In the naturally contaminated wheat, OTA concentrations were highest at 0.95 a_w at 15 °C with mean values of 1592 µg/kg but significantly decreased as temperature increased to 20 °C. However, the highest OTA concentrations in the inoculated wheat were observed at 0.90 a_w at 20 °C. OTA concentrations were generally low at drier conditions of 0.70 and 0.80 a_w at both temperatures in both wheat treatments as shown in Table 5-5.

5.3.5.2 Ochratoxin B

Water activity, storage days, and treatment as individual factors impacted on OTB concentrations. The interaction a_w x temperature, a_w x days, a_w x treatment, temperature x treatment, a_w x temperature x treatment, and a_w x days x treatment influence OTB production in the contaminated wheat. OTB concentrations were highest (60 µg/kg) at 0.95 a_w at 15 °C on day 20 in the naturally contaminated wheat with a significant reduction (15 times) as temperatures increased to 20 °C at the same a_w level and storage day. The highest concentrations of OTB were observed in the inoculated wheat at 0.90 a_w for both temperatures. The mean concentration of OTB was 16 µg/kg at 15 °C, which increased seven times when the temperature changed to 20 °C at the same water activity level (Table 5-5).

5.3.5.3 Ochratoxin alpha

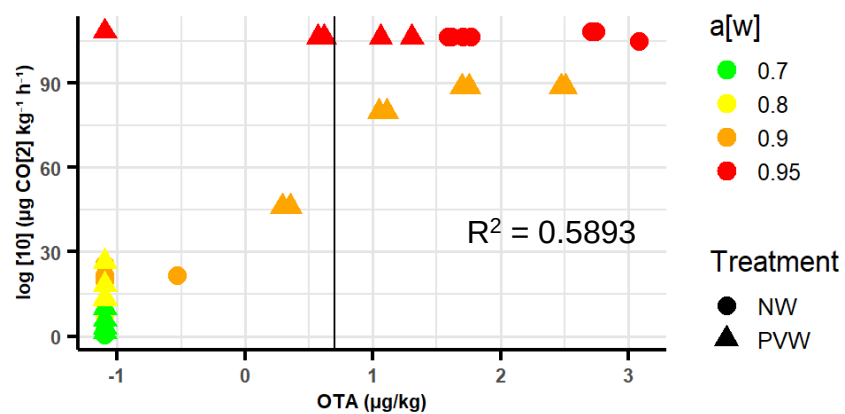
All storage conditions (a_w , days, temperature, and treatment) as single factors significantly impacted the concentrations of Ochratoxin alpha in the contaminated wheat. The interactions between the storage conditions also influence OT α concentrations except for the interactions between temperature x days, temperature x treatment, days x treatment, and a_w x temperature x treatment. The Tukey HSD post hoc test showed that the highest concentration of OT α observed at 0.95 a_w at 15 °C on day 20 (61 µg/kg) was significantly different from

OT α concentrations at other storage conditions in the naturally contaminated wheat.

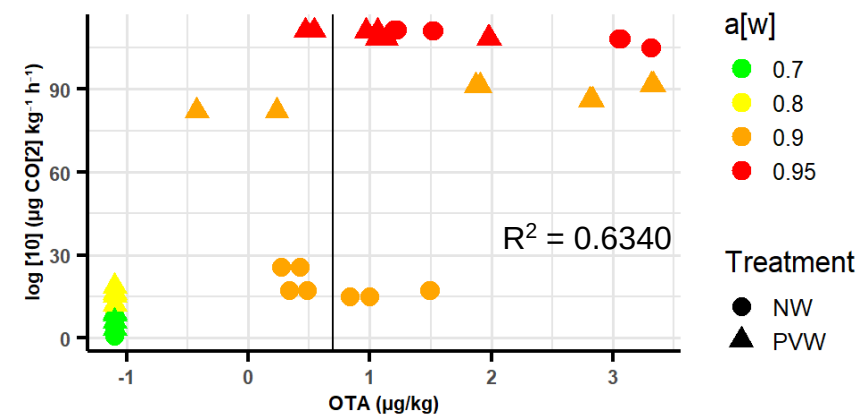
In the inoculated wheat, only temperature significantly impacted the concentrations of OT α . OT α concentrations were mostly below LOD with the highest concentration (2 $\mu\text{g/kg}$) at 0.90 and 0.95 a_w at 20 °C (Table 5-5).

5.3.6 Relationship between respiration rates and OTA in wheat.

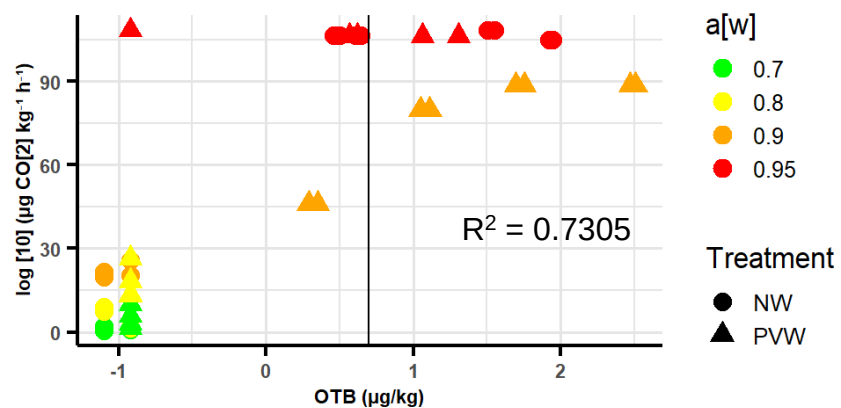
A linear regression model examined the relationship between CO₂ respiration rates and OTA, OTB and OT α in the contaminated wheat. The logarithmic values of each ochratoxin were modelled by temperature, CO₂, a_w , treatment and days. Since storage days were among the strong predictors (with p values of <0.05), separate ggplot graphs were analysed for day 10 and day 20 as shown in Figure 5-2 with their respective adjusted R² values indicating moderate predictions. The model was cross-validated by plotting the predicted ochratoxin values against the known ones (Figure E-2 in Appendix E). The linearity of the plot was not satisfactory. Other regression models such as a generalized linear model and a logistic regression model were performed but were not satisfactory. We attribute this to the non-measurable values (< LOD) of OTA concentrations at drier a_w levels (0.70–0.80 a_w). However, high OTA concentrations were observed at higher a_w (0.90–0.95 a_w) with higher respiration rates having a positive and significant Pearson's correlation coefficient (r) of 0.76. It is therefore important to stress the influence of other storage conditions on the relationship between CO₂ production and OTA concentrations in the wheat. The correlations between CO₂, *Penicillium* populations, and all ochratoxin concentrations are shown in Table E-1 in Appendix E.



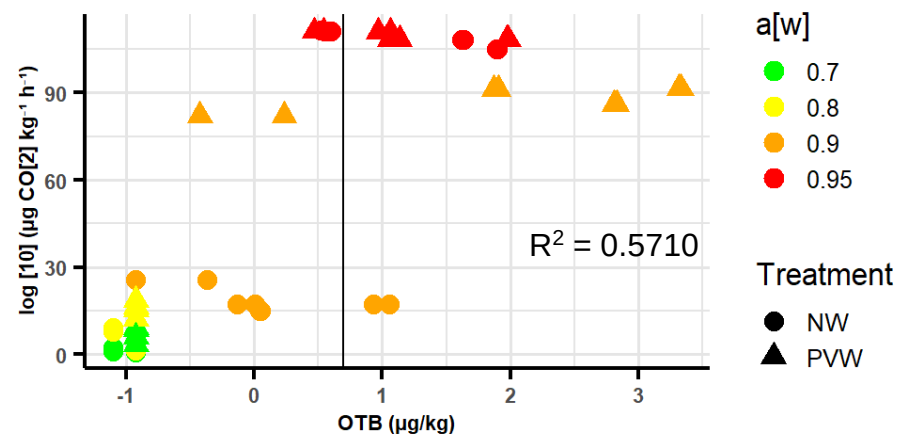
(a) Day 10



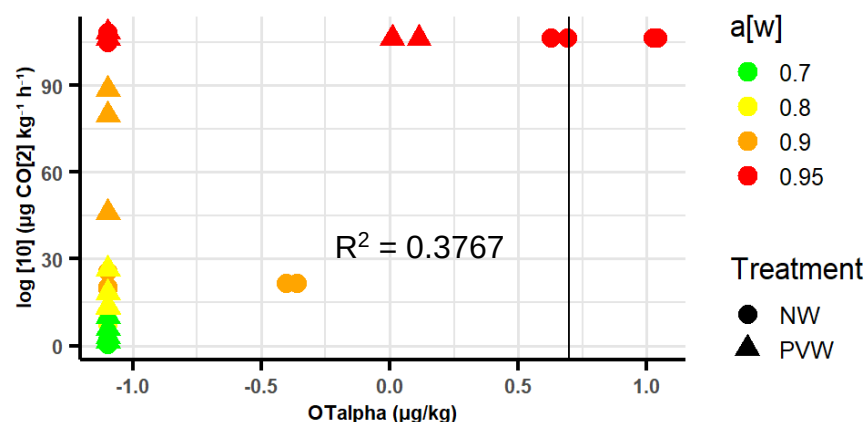
(b) Day 20



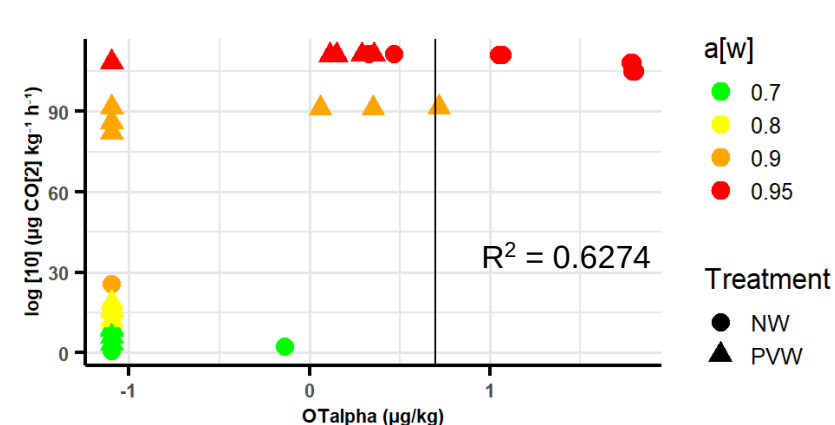
(c) Day 10



(d) Day 20



(e) Day 10



(f) Day 20

Figure 5-3: The relationship between the respiration rates and concentrations of (a) OTA on day 10 (b) OTA on day 20 (c) OTB on day 10 (d) OTB on day 20 (e) OTalpha on day 10 and (f) OTalpha on day 20 at in contaminated wheat at different water activities. Vertical lines indicate maximum levels (5 $\mu\text{g/kg}$) of ochratoxin A concentrations. NW- Naturally contaminated wheat. PVW- *Penicillium verrucosum* inoculated wheat.

Figure 5-3 shows that OTA exceeded the legislative limit at $\geq 0.90 a_w$ (22% moisture content) with $\text{RR} \geq 80 \mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ on day 10, while it exceeded the limit at $\geq 0.90 a_w$ with $\text{RR} \geq 15 \mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ on day 20. This trend was observed for OTB.

For OTalpha, it exceeded OTA's legislative limit at $0.95 a_w$ (25% moisture content) with $\text{RR} \geq 105 \mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ on day 10, while on day 20, OTalpha was above OTA's limit at $\geq 0.90 a_w$ (16% moisture content) with $\text{RR} \geq 90 \mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$.

5.4 Discussion

Moulds and grains respire, producing CO₂, which has been used as an early indicator of mould growth and mycotoxin contamination in stored grains [43,44]. However, the use of CO₂ production to predict the early risk of ochratoxin contamination in wheat under different storage environments is not fully documented. This study employed the use of CO₂ sensors to measure the real-time respiration produced in wheat, examining the prediction of OTA production in wheat stored at 0.70, 0.80, 0.90, 0.95 a_w at 15, and 20 °C to determine the storage conditions where OTA concentrations exceed the legislative limit. The specified water activity levels correspond to the wheat moisture content of 14%, 16%, 22%, and 25% (wet weight basis) [47]. These storage conditions were selected as they are commonly used for storing cereals in temperate zones and promote fungal growth in stored grains.

Water activity and temperature are critical factors that affect mould growth and mycotoxin contamination in foods. Their impacts on the growth of different microbes, especially fungi in various grains, have been widely studied [48–51]. As a xerophilic fungus, *Penicillium* can thrive below < 0.85 a_w, although in temperate climates, it can grow as high as 0.95 a_w. Nevertheless, hydrophilic fungi could outgrow it above 0.95 a_w [48,52,53].

This study shows a significant interaction between water activity and temperature on *Penicillium* populations in naturally contaminated wheat. *Penicillium* populations increased with high water activity and peaked at 15 °C at 0.90–0.95 a_w with no statistical differences between both a_w levels, indicating a 62% increase compared to 0.70 a_w. In a previous study by Cairns Fuller et al. [30], a similar water activity level of 0.95 a_w was reported as the optimal a_w level for *Penicillium* growth in wheat grains, but at 25 °C. Another study reported the growth rate of ochratoxigenic *P. verrucosum* isolates on barley meal extract agar to be highest at 0.95 a_w at 30 °C [54]. Other xerophilic fungal species; *Aspergillus fresenii* and *Aspergillus sulphureus* were reported to grow optimally at 0.94 a_w and 30 °C on Nyjer seeds [37]. However, growth rates in these studies were

monitored by mycelia measurement and not colony counts as used in our research.

To obtain a distinct dominance of *Penicillium* species at the start of the incubation period for adequate growth and OTA production, the wheat grains were inoculated with *P. verrucosum* spores, to have higher CFUs than other fungi on the grain. It was found that the *Penicillium* populations in the inoculated grains were not significantly higher compared to the naturally contaminated grains except at a few conditions (0.80 a_w at 15 °C and 0.95 a_w at 20 °C). Populations increased by 58% at a wet condition of 0.90 a_w compared to populations at the drier condition of 0.70 a_w at 15 °C.

Ochratoxin A, an important storage mycotoxin has gained research attention over the years with more focus on OTA production by *Aspergillus* species, the main OTA producer in grapes and wine. Passamani et al. [55] evaluated the levels of Ochratoxin A produced by *A. carbonarius* and *A. niger* obtained from Brazilian grapes grown in a grape medium in vitro. The study found that the highest concentrations of OTA were observed at 15 °C, 0.99 a_w , and pH 5.35. Another study shows that the highest concentrations of Ochratoxin A produced by two *Aspergillus* strains, *A. carbonarius*, and *A. niger* in maize kernels were observed at 15 °C despite the higher temperatures used in the study [8]. These studies indicate that OTA is optimally produced at a temperature of 15 °C and > 0.94 a_w . However, other researchers have reported that OTA can also be produced optimally at higher temperatures, ranging from 30–37 °C and > 0.90 a_w . A study by Pardo et al. [56] reported the highest OTA concentrations at 0.99 a_w at 30 °C produced by three isolates of *A. ochraceus* in irradiated barley. Lahouar et al. [57] found that *A. tubingensis* produced the highest OTA concentrations in sorghum seeds at 37 °C and 0.97 a_w .

Although storage/ incubation days did not significantly impact the *Penicillium* populations, it had a significant impact on the concentrations of OTA and its interactions with a_w and temperature. Some studies have reported that incubation times affect OTA production, although this is influenced by water availability or temperature changes and substrate type. Chiotta et al. [35] reported OTA

production by *A. carbonarius* at 20 °C and 0.950–0.965 a_w , within 14 days of incubation in synthetic grape juice medium. Meanwhile, *A. ochraceus* produced the highest amount of OTA on Corinth's raisins on the 24th day of incubation [58] and both *A. niger* and *A. carbonarius* produced OTA from the 5th day of incubation in maize kernels [8]. The variation in OTA production time may be due to indigenous microflora found in various substrates which can either detoxify OTA or reduce its production. This study only analyzed OTA levels on day 10 and day 20 and not on a series of days. The concentrations on day 10 were mostly below the limit of detection (LOD) and were significantly different from those on day 20. Due to variations in LOD values and fungal species, it is difficult to make direct comparisons between OTA concentrations in our study to those in other studies. Generally, at wet conditions (0.90–0.95 a_w), OTA concentrations were significantly higher than OTB and OT α concentrations. At, 15 °C, OTA concentrations were twenty-six times higher than its dechlorinated (OTB) and less potent variant (OT α) in the naturally contaminated wheat.

The byproducts of the respiration of grain and its natural microbiota are carbon dioxide, water vapour, and heat. Lower RR were observed in the wheat at drier conditions while higher water activity levels had higher RR. This was supported by the findings of Huang et al. [59] and Dillahunty et al. [60] on the effect of a_w on respiration rates in corn and rice respectively. The CO₂ sensors, used to measure carbon dioxide concentrations, have a maximum measuring capacity of 50010 ppm. Once the limit was reached, the readings remained constant throughout storage. Hence, in both contaminated wheat, samples at the wettest water activity (0.95 a_w) had the same respiration rates on day 10 and day 20. During the initial phase of the experiment, the respiratory rate (RR) remained low and stable for a few days before increasing. However, the RR increased as the temperature and water activity level increased. This finding contradicts the observation of Raudiene et al. [42], who reported high RR at the beginning of their experiment because the grains used were not equilibrated. Therefore, after grain rehydration, the RR was high due to water on the grain surface. However, as water infiltrated the grains, the respiration process decelerated and was influenced by alterations in temperature and water availability.

Surprisingly, *Penicillium* populations were negatively correlated with RR and Ochratoxin concentrations in the naturally contaminated wheat (Table E-1 in Appendix E). The highest RR was observed at 0.95 a_w at 20 °C where *Penicillium* populations and OTA concentrations were low. However, the increase in RR due to fungal activity indicates the start of grain deterioration which can be examined visually or with a stereoscope as shown in Figure 5-1. The presence of other fungal genera found in the contaminated wheat grains; *Aspergillus*, *Fusarium*, *Aspergillus* section *Aspergillus* (previously called *Eurotium*), *Cladosporium*, *Mucor*, *Rhizopus*, and *Alternaria* may contribute to the increased RR. Other fungal genera were also impacted by changes in water and temperature in the stored wheat.

Temperature did not significantly impact the RR, but a significant difference exists between RR in both wheat treatments. Although the relationship between the CO₂ RR and ochratoxin concentrations at drier a_w levels was not fully established due to low measurable values (< LOD) of OTA, our observation is that, at a higher a_w level, RR was high and ochratoxin concentrations were high. Therefore, the knowledge of optimal and marginal levels of ecological factors including the CO₂ production within a grain silo could significantly improve the storage management of grains.

5.5 Conclusion

Grain quality can quickly deteriorate during storage due to environmental factors such as moisture and temperature. If storage is not properly managed, mycotoxins can spread from a hotspot area to the entire grain mass in the silo. To monitor this, CO₂ sensors integrated with relative humidity (RH) and temperature sensors were installed in the wheat silos and coupled to a decision support system (DSS). This linked the real-time RR data to biological boundary models for moisture content and temperature conditions which were either favourable or unfavourable for the growth of *Penicillium verrucosum* and ochratoxin production in the wheat. This study found that CO₂ RR in stored wheat was significantly affected by changes in grain moisture and temperature. It also

established the relationship between CO₂ RR and ochratoxin concentrations at the wettest conditions. Installing such real-time systems will allow the identification of the areas within silos that may represent hot spots and improve the pro-activeness of grain managers to take remedial action, to reduce food waste.

5.6 References

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6 Real-time CO₂ production monitoring in stored oats as an indicator of type A trichothecenes and ochratoxin A contamination under simulated environmental conditions.

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Abstract

Grain industries are interested in an integrated approach to in-silo grain quality and safety management using carbon dioxide measurement together with temperature and moisture monitoring. This study examines if CO₂ production could predict mycotoxin production (T-2 toxin, HT-2 toxin, its glucoside, and ochratoxin A (OTA)) and identify storage conditions exceeding legislative limits in stored oats for the first time. The impact of water activity (a_w) levels (0.70–0.95 a_w), temperature (15 and 20 °C), and storage duration on (a) *Fusarium* and *Penicillium* populations, (b) CO₂ respiration rates (RR), and (c) mycotoxin concentrations in stored oats was investigated. One hundred and twenty samples were analysed for multiple mycotoxins by LC-MS/MS. Substantial differences were found in the RR of oats at $\geq 0.90 a_w$ at both temperatures. A moderate positive correlation between CO₂ and mycotoxins was observed, and mycotoxins exceeded their limits at $\geq 0.80 a_w$ (16% moisture content) when $RR \geq 25 \mu\text{g CO}_2 \text{ kg}^{-1}\text{h}^{-1}$. This knowledge forms the basis for developing decision support systems for improving oats storage management.

Keywords: *Fusarium langsethiae*, T-2/HT-2 toxin, ochratoxin A, carbon dioxide respiration rate, water activity, LC-MS/MS.

6.1 Introduction

Oats (*Avena sativa* L.) are significant cereal crops highly valued for their dietary benefits and growing market demand with the global oats market projected to reach \$10.8 billion by 2032 [1]. It is well-suited to cooler environments, requiring an optimal temperature range of 15–25 °C and moist conditions [2]. However, post-harvest losses due to spoilage, primarily caused by moulds and consecutive mycotoxin contamination, remain a significant challenge contributing to around 14% of food produced globally [3]. Mycotoxins are toxic compounds produced by certain fungi under specific environmental conditions. The accumulation of mycotoxins in oats poses both economic concerns and health risks. [4].

Fungal species such as *Fusarium*, *Penicillium*, and *Aspergillus* are commonly linked to oat spoilage and diseases, including *Fusarium* head blight [5–9]. These moulds thrive in warm and humid storage conditions and reduce the quality and quantity of grains producing harmful mycotoxins. *Fusarium langsethiae* is one of the most important *Fusarium* species found in oats [10]. It produces type A trichothecenes (T-2/HT-2 toxins). Other fungal genera produce ochratoxin A (OTA), deoxynivalenol (DON), and fumonisins [11–14].

To mitigate the risks of mycotoxins, various countries and international organisations have established regulations and guidelines to control mycotoxin levels in food and feed. These regulations aim to ensure food safety and protect consumer health by setting maximum permissible levels of mycotoxins in various commodities. In the European Union, the maximum level for the sum of T-2+HT-2 in unprocessed oats was enforced in 2024 and is 1250 µg/kg [15].

Respiration in stored cereal grains is the metabolic process of breaking down stored carbohydrates, such as starch, into simpler compounds like carbon dioxide (CO₂) and water, releasing energy [16]. This process is influenced by abiotic factors (such as moisture, and temperature), oxygen availability, and grain condition in storage [17]. Therefore, CO₂ produced in stored grain mass is due to the respiration of the grains and the biotic factors present such as microorganisms and insects. Several studies have addressed the impact of

abiotic factors on the growth and mycotoxin production of *Fusarium* species [18–20].

As the moisture content (MC) rises in the grain silo, so does the temperature. The temperature of the external environment affects the silo wall, leading to condensation within the silo when the temperature decreases. This, in turn, increases the moisture content in the grains, causing them to respire and elevating the temperature inside the silo, ultimately resulting in grain spoilage. The movement of moisture and CO₂ concentrations within a bulk of grain mass was described by Ramachandran, [21].

Due to the inter-relationship between MC, temperature and gas produced in grain silos, it is important to adopt an integrated approach that combines the monitoring of moisture, temperature and CO₂ concentrations in stored grain to improve grain quality and safety in storage. Some studies have employed this approach [22–25]. However, these studies did not consider the relationship between these abiotic factors (MC x temperature x CO₂) and mycotoxins produced in the grains. Mylona et al. [26] assessed fungal growth and dry matter loss (DML) with the amount of CO₂ produced in stored irradiated wheat and maize under different interacting conditions of water activity (a_w) and temperature. They reported increased respiration rates as a_w and temperature increased with varying production patterns of *Fusarium* species. However, gas measurements in the irradiated grains were conducted by gas chromatography (GC). This does not accurately reflect the real-time CO₂ measurements of the natural mycobiota of non-irradiated grains. Garcia-Cela et al. [27] also reported that respiration rates and related DMLs can indicate the relative likelihood of mycotoxin contamination in natural and irradiated wheat grains. The respiration rates were also measured with GC and the grain sample size (10 g) is considerably small compared to the study of Oluwakayode et al. [28]. They investigated the relationship between the real-time CO₂ measurement with CO₂ sensors and ochratoxins concentrations produced in a mini silo of wheat grains under different a_w and temperature conditions. Similar to previous studies, respiration rates increased as a_w increased and OTA concentrations were highest at the wettest a_w level (0.95 a_w).

They highlighted a positive correlation between the CO₂ and OTA produced at the wettest storage conditions.

This study aims to investigate the relationship between the respiration rates, type A trichothecenes and OTA concentrations in mini silos of oats under different interacting environmental factors. We wanted to determine if CO₂ production could indicate the risk of these mycotoxin contaminations above legislative limits in stored oats.

The objectives are to investigate the effect of storage conditions (0.70, 0.90, and 0.95 a_w at 15 and 20°C) on (a) mycotoxigenic fungal populations (b) CO₂ respiration rates (RR) (c) the sum of T-2/HT-2 toxin+ HT-2- glucoside and OTA produced in naturally contaminated and *Fusarium langsethiae* inoculated oats and (d) to examine the relationship between the CO₂ and mycotoxins concentrations in the contaminated oat.

6.2 Materials and Methodology

6.2.1 Oat grains and moisture adsorption curve analysis

Mascani oats harvested in England (2022 harvest) were stored at 4 °C for 2 weeks before the experiment. AquaLab water activity meter 4TE Decagon devices, (Inc, Pullman, USA) was used to analyse the initial a_w of grains. The grains' moisture content (MC) was analysed by drying grains in the oven at 105 °C overnight. The MC values were calculated on a dry weight basis. The moisture adsorption curve (MAC) was adapted from Oluwakayode et al. [14]. The relationship between the MC (dry weight basis) and a_w values was plotted and noted and the MAC for the natural oats is found in Figure F-1 in Appendix F.

6.2.2 Fungal strain and spores harvesting

Fusarium langsethiae (2390/2391) known to produce T-2 toxin and HT-2 toxin and previously used by Medina et al. [18] was used in this study. The strain was stored at -20°C in glycerol: water (70:30, v/v) in the Magan Centre of Applied Mycology culture collection, Cranfield University. *Fusarium langsethiae* was grown on Malt Extract Agar (MEA) with an anti-bacterial agent (chloramphenicol) and incubated at 25°C for 7 days. The spores harvesting method was adapted from Oluwakayode et al. [28].

6.2.3 Mini-silo CO₂ sensing storage experiment.

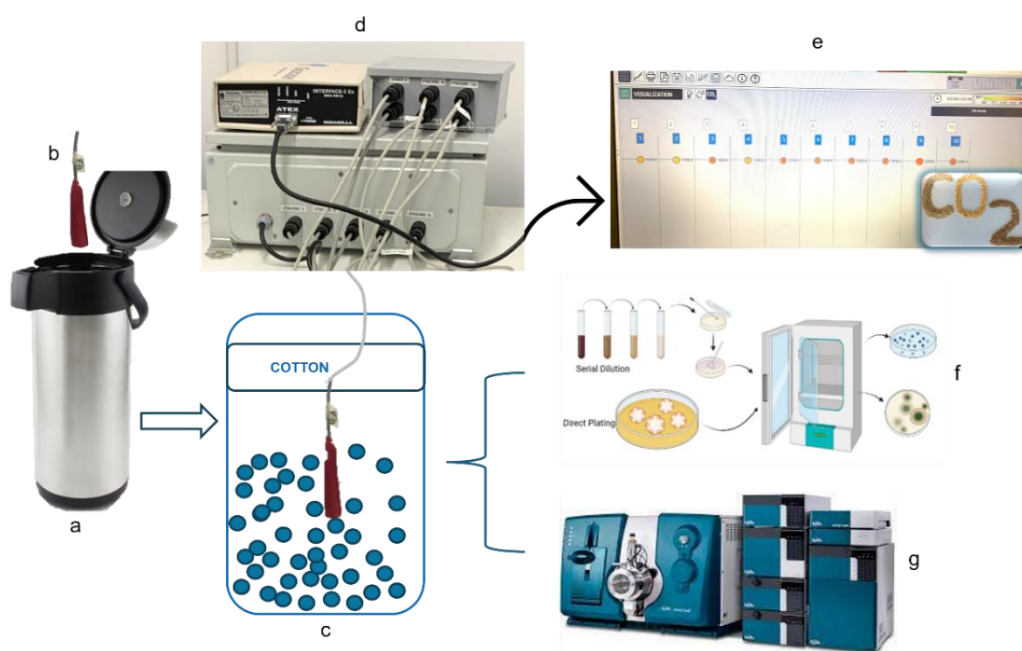


Figure 6-1: Experimental design of the mini silo of CO₂ sensing in stored oats. (a) 5 Litre coffee flask (VonShef, Manchester, UK) (b) CO₂ sensor, (Gescaser, Spain) (c) oats and sensor and cotton inside the coffee flask (d) multiplex sensor control box (e) system collecting CO₂ signals (f) scheme of the fungal enumeration analysis (g) mycotoxins analysis with Liquid Chromatography Tandem-Mass spectrometer (LC-MS/MS), (SCIEX, USA).

Targeted water activities (0.70, 0.90, 0.95 a_w) were achieved in 2.5 kg of oat grains weighed into a 12-litre box mixed with a known amount of water and 20

mL less for spore inoculum for inoculation in the 0.90 and 0.95 a_w grains. The grains were vigorously mixed and stored at 4 °C overnight. Grains were then equilibrated at the targeted temperature (15 and 20 °C) rooms for 3 hours, and the equilibrated grains' a_w were measured. Grains were transferred into a coffee flask with a centrally placed ATEX-compliant sensor probe. Gaseous movement in and out of the flask was minimised by placing cotton wool in the head-space of the flasks. There were three flasks (replicates) for each a_w level and the nine flasks were stored at each temperature condition. The probe is made of CO₂, temperature, and relative humidity sensors connected to a computer system where their respective real-time monitoring readings from the stored grains were collected for 20 days. Sixty grams of contaminated samples were collected on days 10 and 20 from each flask with a sterile probe into sterile jars. Multiple small sizes of grains were taken from different spots to have a good representation of the bulk sample. The a_w of the grains was monitored before and after sampling. Twenty grams of the sub-sample grains were analysed for fungal populations and isolations while the remaining forty grams were oven-dried at 50–55 °C overnight, ground, and stored at –20 °C before further analysis. The CO₂ sensors were calibrated before each experiment for each treatment and temperature studied. After sampling on day 10, the grain inside the flask is sixty grams less, the new weight is considered for calculating the respiration rates for day 20. The respiration rate was calculated using the diffusion formula in Equation 6-1 and 6-2 adapted from Raudienė et al. [25].

$$\text{Respiration rates} = \frac{\Delta\text{CO}_2 \times M_{\text{CO}_2} \times V_h}{V_m \times m \times \Delta t} \quad (6-1)$$

where ΔCO_2 is the change in CO₂ volumetric concentration in ppm, M_{CO_2} is the molar mass of CO₂ gas = 44.01 (gmol⁻¹), V_h is the volume of headspace in the jar (L), V_m is the molar volume of a gas (Lmol⁻¹), m is the mass of the oat sample (kg) and Δt is the duration (h) of ΔCO_2 .

The molar volume was determined by analyzing the storage temperatures.

$$V_m = \frac{R \times T}{P} \quad (6-2)$$

where R is the gas constant (L mol⁻¹ K⁻¹), T is temperature (K), P is pressure (atm).

6.2.4 Fungal population and isolation

6.2.4.1 Fungi populations

According to the manufacturer's instructions, 15 g of peptone agar was mixed in one litre of distilled water. 9 mL was dispensed into 25 mL universal glass bottles, autoclaved at 121 °C for 15 minutes, and allowed to cool.

One gram of contaminated oats kernels was soaked in sterile 9 mL peptone water in a 25 mL universal bottle for 3 hours. One mL of this bottle was serially diluted (by ten-fold dilution) to the 0.0001M solution bottle. Sterile tips were used between each dilution and the universal bottle's content was shaken using a vortex. 100 µL of the dilution aliquots were reversely plated onto Dichloran Glycerol 18 agar (DG18+: Oxoid CM0729) and Malt Extract Agar (MEA+: Oxoid CM59) media and Malt Extract Agar (MEA+: Oxoid CM59) media in 3 replicates and spread out with a sterile metal spreader. The plates were incubated at 25 °C for 7 days. Fungi genera were identified visually with a stereoscope (Olympus 308136, Wild M7A, Heerbrugg, Switzerland) and morphological characteristics [29]. Colonies were counted with a colony counter (Gallenkamp, Cambridge UK) and expressed as log₁₀ CFUs/g-dry weight.

6.2.4.2 Fungi isolations

To identify the dominant fungal genera in the contaminated grains, 25 grains were directly inoculated on DG18+ and MEA + media. Five grains were placed

equidistant on each medium plate of five replicates and incubated at 25 °C for 7 days. Fungal genera were identified visually with a stereoscope and morphological characteristics. The frequency of isolation was calculated using Equation 6-3 below.

$$\% \text{ Frequency of isolation} = \frac{\text{Total contaminated grains}}{\text{Total grains in plates (25)}} \times 100 \quad (6-3)$$

6.2.5 Mycotoxin analysis

6.2.5.1 Chemical reagents

T-2 toxin, HT-2 toxin, and OTA standard solutions were purchased from Romer Labs (Tulln, Austria), and HT-2-toxin-3-O-β-D-glucoside was synthesized according to Michlmayr et al. [30]. LC-MS/MS-grade methanol, acetonitrile (ACN), and formic acid (Honeywell, Seelze, Germany); ammonium acetate (MS grade, Sigma-Aldrich, Darmstadt, Germany); and glacial acetic acid (HAc, Sigma-Aldrich, Burlington, MA, USA) were used. Water was purified successively by reverse osmosis and using a Pure LAB water system.

6.2.5.2 LC-MS/MS

The liquid chromatography tandem mass spectrometry (LC-MS/MS) machine used in this study was a LQTRAP 5500+ MS/MS system (SCIEX) equipped with a Turbo V electrospray ionization (ESI) source, coupled to an ExionLC AD System (SCIEX, Framingham, MA, USA). Chromatographic separation was performed at 27 °C on a Gemini C18-column, 100 × 4.6 mm (Phenomenex, Torrance, CA, USA).

The LC-MS/MS was performed in the time-scheduled MRM mode both in positive and negative polarities in one chromatographic run per sample by scanning two fragmentation reactions per analyte. Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM of ammonium acetate and were composed of water/methanol/acetic acid at 89:10:1 (v/v/v; eluent A) and 2:97:1

(v/v/v; eluent B), respectively, with a sample injection volume set at 5 μ L, with a total runtime of 21 minutes. The gradient elution program for the elution of mycotoxins was as follows: 0 min 5% B, 0.5 min 5% B, 2.5 min 70% B, 3.5 min 95% B, 5 min 95% B, 7.5 min 5% B. The mass spectrometry parameters used are outlined in Table 6-1.

Table 6-1: Optimised MS/MS parameters for the analysed mycotoxins, including precursor ions, product ions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP).

Components	Precursor Ion (<i>m/z</i>)	Product Ion (<i>m/z</i>)	DP (V)	CE (V)	CXP (V)
T-2 toxin	484.3	215.2	57	29	17
	484.3	185.1	27	33	11
HT-2 toxin	447.4	345.1	131	27	20
	442.2	323.2	50	15	16
HT-2-glucoside	604.3	323.1	101	17	16
	604.3	263.1	101	23	14
Ochratoxin A	404.0	239.0	91	37	16
	404.0	102.0	102	105	14

6.2.5.3 Method validation

The acceptable performance criteria of analytical methods set and updated by European Commission regulation No. 2021/808/EC [31] were used to validate the optimised LC-MS/MS method for mycotoxin analysis in the oat samples. The performance characteristics evaluated were linearity (r^2), limit of detection (LOD), limit of quantification (LOQ), matrix effect expressed as signal suppression/enhancement (SSE), recovery of the extraction process (RE), absolute recovery (RA), and repeatability.

Working standard solutions were prepared to contain 25 μ L each of stock solutions of T-2 toxin, HT-2 toxin, HT-2-glucoside and OTA mixed in ACN

solution. 50 mg of three lots of homogenised oat samples were spiked at the working solution concentration and a 10-fold dilution level. This was done in duplicates. Spiked samples were placed in the dark overnight for solvent evaporation and analyte-matrix interaction. The spiked and non-spiked samples were extracted with ACN: H₂O: HAc (79: 20:1, v/v/v) for 90 minutes using a VWR DVX-2500 shaker (VMR International Ltd Leicestershire, UK). After extraction, the extract of the non-spiked samples was spiked and 500 µL of the extract was diluted with 500 µL of the diluent ACN: H₂O: HAc (20:79:1, v/v/v). 5 µL of each diluted extract was injected twice into the LC-MS/MS system for analysis. 500 µL of the working standard solution was diluted with ACN: H₂O (50:50, v/v) to achieve the desired concentration of each analyte in the working standard. Quantification was performed via external calibration using a nine-point calibration curve achieved by serial dilutions of the multi-analyte working standard solution. Data were further processed using Analyst® 1.7.1 and SCIEX OS-Q 3.0.

The apparent recovery (RA), matrix effect (SSE), and extraction efficiency (RE) were calculated using Equations 4–6 below.

$$RA (\%) = \frac{\text{area (sample spiked before extraction)}}{\text{area (neat solvent standard)}} \times 100 \quad (6-4)$$

$$SSE (\%) = \frac{\text{area (sample spiked after extraction)}}{\text{area (neat solvent standard)}} \times 100 \quad (6-5)$$

$$RE (\%) = \frac{RA}{SSE} \times 100 \quad (6-6)$$

6.2.5.4 Sample preparation and extraction

The initial mycotoxin concentrations of the oat grains were analysed. Following the storage experiment, 120 contaminated oat samples (including replicates)

were analysed for mycotoxins. The oats were ground with a high-power 1000W blender (Geroge, Watford, UK). A volume of 20 mL of extraction solvent (ACN: H₂O: HAc, 79:20:1, v/v/v) was added to 5 g of ground oat grains. Extraction was carried out for 90 min using a multitube vortex (VWR DVX-2500, VMR International Ltd Leicestershire, UK), followed by centrifugation for 15 minutes at 5000 rpm on a Rotina 380R centrifuge (Hettich, Tuttlingen, Germany). Then, 500 µL of the extract was diluted with 500 µL of dilution solvent (ACN/H₂O/acetic acid, 20:79:1, v/v/v). 5 µL of the diluted extract was injected into the LC-MS/MS system for analysis.

6.2.6 Statistical analyses

The data sets were analysed with Statistica 14.0.1, JMP® Pro 17, Microsoft Excel, and RStudio in R 4.4.1 version. The normal distribution of data was examined with the normal plots of the residuals. When data failed the normality test, data were transformed to square root or logarithm values to achieve normality. Transformed data were normally distributed; therefore, factorial ANOVA was used to analyze the effect of the interactions between the storage conditions on respiration rates and mycotoxin concentrations. The Tukey Honest Significant Difference (HSD) post hoc test was used to evaluate differences among means for the interactions of storage conditions. The non-parametric comparison for each pair (Wilcoxon method), evaluates significant differences in fungal populations and mycotoxin concentrations for each storage condition. Statistical analyses performed were considered significant when p values were <0.05. The concentrations of mycotoxins below the limit of detection and quantitation were assigned with values of LOD/2 and LOQ/2, respectively, for statistical analysis [32].

6.3 RESULTS

6.3.1 Visual representation of contaminated oats.

Figure 6-2 shows contaminated oats with visible mould growth from the silos. Mould spots were observed more at 0.95 a_w than at 0.90 a_w . Moulds were not visible at the 0.70 a_w . The pictorial representations of fungal populations or isolations in the naturally contaminated oats on MEA+ media at all a_w levels at 15°C are shown in Figure F-2 Appendix F.

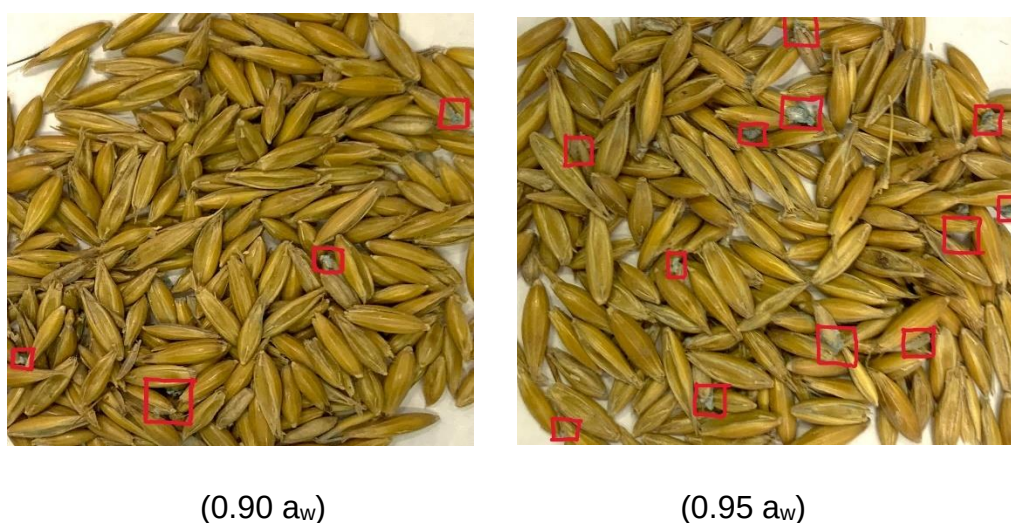


Figure 6-2: Contaminated oats with mould spots after storing for 20 days at 15 °C.

6.3.2 Fungal populations and isolations in contaminated oats.

Tables 6-2 and Table 6-3 show the mean values of the colony-forming units and the frequency of isolation of potentially mycotoxigenic fungal genera in both oat treatments per media.

Table 6-2: Mean values ($n = 9 \pm \text{SD}$) of *Fusarium* and *Penicillium* populations ($\log_{10}$ CFUs/g) in contaminated grains at all storage conditions.

Fungal genera	Grains	T (°C)	Days	0.70 a_w		0.90 a_w		0.95 a_w	
				MEA+	DG18+	MEA+	DG18+	MEA+	DG18+
<i>Fusarium</i>	NO	15	10	ng	ng	ng	2.9 ± 2.7	ng	ng
			20	3.3 ± 3.4	2.1 ± 2.3	3.3 ± 3.3	2.7 ± 2.6	ng	ng
		20	10	ng	2.8 ± 2.8	2.1 ± 2.3	2.6 ± 2.5	6.2 ± 6.4	ng
			20	4.1 ± 4.0	ng	ng	ng	ng	ng
	FLO	15	10	ng	ng	ng	2.1 ± 2.3	ng	ng
			20	ng	ng	3.2 ± 3.3	ng	ng	ng
		20	10	2.9 ± 2.8	2.4 ± 2.6	3.9 ± 4.0	ng	ng	ng
			20	3.3 ± 3.3	1.5 ± 1.6	3.3 ± 3.3	ng	ng	ng
<i>Penicillium</i>	NO	15	10	3.1 ± 2.9	ng	2.9 ± 3.1	2.8 ± 2.7	6.7 ± 6.2	6.8 ± 0.0
			20	2.9 ± 0.0	2.1 ± 2.3	3.3 ± 3.4	2.9 ± 3.0	7.0 ± 6.6	6.9 ± 6.4
		20	10	3.5 ± 3.4	3.6 ± 3.5	2.9 ± 3.0	3.2 ± 3.1	6.9 ± 6.2	6.9 ± 6.1
			20	3.1 ± 2.8	2.9 ± 2.6	5.3 ± 5.2	5.6 ± 0.0	7.0 ± 6.9	7.0 ± 6.9
	FLO	15	10	3.2 ± 3.2	3.2 ± 2.9	3.0 ± 3.0	3.2 ± 2.6	6.7 ± 6.0	6.8 ± 6.2
			20	3.8 ± 4.0	3.7 ± 3.9	2.8 ± 2.7	2.9 ± 3.0	7.2 ± 6.7	7.0 ± 7.1
		20	10	2.8 ± 2.6	3.1 ± 2.7	3.7 ± 3.3	4.0 ± 3.9	6.8 ± 6.6	6.8 ± 6.2
			20	3.8 ± 4.0	3.7 ± 3.9	2.8 ± 2.7	2.9 ± 3.0	7.2 ± 6.7	7.1 ± 7.1

SD: Standard deviation. NO: Naturally contaminated oats. FLO: *Fusarium langsethiae* inoculated oats. T: Temperature. a_w : water activity. DG18+: Dichloran Glycerol 18 agar + Chloramphenicol. MEA+: Malt Extract Agar + Chloramphenicol. ng: no growth. Non-parametric comparison for each pair using the Wilcoxon method shows no significant differences in the fungal populations among the three a_w levels for each media for each day and each temperature (within each row). LOD is $1000 \text{ CFU/g} = 3.0$ (logarithmic value).

Fusarium populations were mostly observed at 0.70 and 0.90 a_w , while *Penicillium* populations were mostly present at all storage conditions.

Aspergillus sect. *Nigri* and *Aspergillus* sect. *Flavi* were only present at 0.95 a_w in the naturally contaminated oats at 20°C. *A.* sect. *Nigri* mean values are 5.9 ± 6.1 CFUs/g and 5.9 ± 6.0 CFUs/g on MEA+ and DG18 + respectively while *A.* sect. *Flavi* mean values are 5.1 ± 5.3 CFUs/g and 5.2 ± 5.4 CFUs/g on MEA+ and DG18 + respectively.

Table 6-3: Frequency of isolation (%) of *Fusarium*, *Penicillium*, and *Aspergillus* in contaminated grains at all storage conditions.

Fungal genera	Grains	T (°C)	Days	0.70 a_w		0.90 a_w		0.95 a_w	
				MEA+	DG18+	MEA+	DG18+	MEA+	DG18+
<i>Fusarium</i>	NO	15	10	7	45	12	24	-	-
			20	4	43	15	20	11	-
		20	10	-	49	-	39	11	-
			20	-	49	-	64	1	-
	FLO	15	10	-	29	-	32	-	-
			20	-	29	-	28	-	-
		20	10	-	56	-	36	44	-
			20	1	36	1	16	-	-
<i>Penicillium</i>	NO	15	10	85	96	83	96	100	100
			20	77	87	81	91	100	100
		20	10	79	99	81	97	89	100
			20	72	97	69	97	68	96
	FLO	15	10	72	97	59	85	100	100
			20	71	95	68	99	100	100
		20	10	68	99	68	97	76	88
			20	72	56	79	79	33	33
<i>Aspergillus</i> . sect. <i>Nigri</i>	NO	15	10	-	3	-	-	-	-
			20	3	1	-	-	-	-
		20	10	-	-	-	1	17	9
			20	3	-	-	1	33	29
	FLO	15	10	-	4	-	-	-	-
			20	-	-	-	-	-	-
		20	10	-	-	-	-	-	-
			10	-	-	-	-	-	-

Fungal genera	Grains	T (°C)	Days	0.70 a _w		0.90 a _w		0.95 a _w	
				MEA+	DG18+	MEA+	DG18+	MEA+	DG18+
			20	1	-	-	-	-	13
<i>Aspergillus</i> sect. <i>Flavi</i>	NO	15	10	-	-	-	-	-	-
			20	-	-	-	-	-	-
		20	10	-	-	-	-	51	29
			20	-	-	-	-	73	71
	FLO	15	10	-	-	-	-	-	-
			20	-	-	-	-	-	-
		20	10	-	-	-	-	4	-
			20	-	-	-	-	33	52

NO: Naturally contaminated oat. FLO: *Fusarium langsethiae* inoculated oats. T: Temperature. a_w: water activity. DG18+: Dichloran Glycerol 18 agar + Chloramphenicol. MEA+: Malt Extract Agar + Chloramphenicol.

Fusarium isolation was higher in DG18+ media than in MEA+ at 0.70 and 0.90 a_w. but absent in the 0.95 a_w on DG18+ media. Of the potentially mycotoxigenic fungal strain, *Penicillium* was relatively abundant in both treatments and media and highest at 0.95 a_w. It is interesting to note that *Aspergillus* species were relatively low at drier storage conditions but present at 0.95 a_w especially at 20 °C where *Fusarium* growth was least observed.

6.3.3 The influence of storage conditions on respiration rates in contaminated oats.

The respiration rates (RR) increased significantly ($p < 0.05$) as the a_w increased in the stored oats (Table 6-4). An increase in storage days and treatment type as single factors impacted RR significantly. However, temperature rise did not have any significant effect on RR ($p > 0.05$). The mean values of respiration rates ($\mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$) in contaminated oats at all storage conditions are shown in Table 6-4. In the naturally contaminated stored oats, RR was more than nine times lower at 0.70 a_w compared to the wet a_w level at 15 °C, while it was more than fourteen times lower at 0.70 a_w compared to wet conditions at 20 °C.

The interactions of storage conditions on the RR in contaminated oats are shown in Table 6-5. Figure F-3 in Appendix F shows the respiration rates produced at day 0, day 10 and day 20 for each a_w , temperature and treatment.

Table 6-4: Mean values ($n=3 \pm SD$) of respiration rates ($\mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$) in contaminated oats.

Grains	T (°C)	Days	0.70 a_w	0.90 a_w	0.95 a_w
Naturally contaminated oat	15	0	1.9 ± 0.3^a	8.4 ± 0.5^b	11.5 ± 2.3^c
		10	1.9 ± 0.6^a	34.2 ± 0.6^b	127.2 ± 4.0^c
		20	2.1 ± 0.6^a	29.8 ± 1.9^b	135.0 ± 0.1^c
	20	0	1.3 ± 0.4^a	10.8 ± 1.0^b	11.7 ± 2.3^b
		10	2.0 ± 1.0^a	63.0 ± 11.7^b	133.0 ± 0.0^c
		20	3.4 ± 2.6^a	48.0 ± 11.1^b	111.2 ± 28.4^c
<i>F. langsethiae</i> inoculated oat	15	0	2.3 ± 0.3^a	7.2 ± 0.3^b	11.4 ± 2.4^b
		10	1.6 ± 0.4^a	36.0 ± 3.2^b	131.2 ± 0.0^c
		20	1.8 ± 0.4^a	32.4 ± 1.5^b	134.9 ± 0.1^c
	20	0	0.9 ± 0.1^a	7.1 ± 0.2^b	8.0 ± 0.2^b
		10	0.8 ± 0.1^a	36.0 ± 1.9^b	131.1 ± 1.9^c
		20	0.8 ± 0.1^a	24.0 ± 1.9^b	124.2 ± 13.3^c

SD- Standard deviation. T- Temperature. a_w - water activity. T- Temperature. a_w - water activity. Different letters show significant differences in the RR for each a_w and days in each row.

Table 6-5: The effect of the interactions of storage conditions and their p values on respiration rates in contaminated oats

Interaction effect	P values
Treatment	0.027164
a_w	0.000000
Temp	0.790288
Days	0.040745
Treatment* a_w	0.002177
Treatment*Temp	0.007701
a_w *Temp	0.002228
Treatment*Days	0.776132
a_w *Days	0.077652
Temp*Days	0.049136
Treatment* a_w *Temp	0.000574
Treatment* a_w *Days	0.788136
Treatment*Temp*Days	0.711080
a_w *Temp*Days	0.331322

Treatment*a_w*Temp*Days	0.525270
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a_w-water activity. Temp- temperature

6.3.4 Method validation performance in the oat matrix

The extraction efficiency (R_E) of each analyte was within the acceptable range (70–120%) according to the amended guideline set by European Commission regulation No. 2021/808/EC [31]. The goodness of fit of the calibration curve for each analyte was acceptable, with r^2 values (coefficient of determination) of > 0.990 . The relative standard deviation (RSD %) was satisfactory for the validated analytes $<14\%$ except for OTA (17%). The relative standard deviation of the within-laboratory reproducibility (RSD_{WLR}), the matrix effect or the signal suppression/ enhancement (SSE), and the apparent recovery (R_A %) were calculated from the average of 48 replicates of the three different lots of oats spiked in quadruplicate across three separate days. The limit of detection (LOD) and limit of quantitation (LOQ) for the analytes ranged from 0.8- 4.1 $\mu\text{g/kg}$ and 2.6-13.6 $\mu\text{g/kg}$ respectively, which were lower than the minimum acceptable levels for the regulated mycotoxins in unprocessed oats [33,34]. The mean values of the R_A , R_E , RSD, LOD, and LOQ for each analyte are shown in Table 6-6.

Table 6-6: Mean values (n = 48) of the inter-day precision within-lab reproducibility (WLR) method validation for oat grains.

Components	R_A (%)	RSD (%)	R_E (%)	SSE (%)	LOD ($\mu\text{g/kg}$)	LOQ ($\mu\text{g/kg}$)
HT-2 Toxin	88	7	92	96	4.1	13.6
HT-2-glucoside	97	6	104	93	3.9	13.2
T-2 Toxin	81	9	79	102	3.7	12.4
Ochratoxin A	97	17	105	92	0.9	2.9

R_A —apparent recovery, R_E —extraction efficiency, RSD—relative standard deviation, SSE—signal suppression/enhancement, LOD—limit of detection, LOQ—limit of quantitation.

6.3.5 The influence of the interactions of storage conditions on mycotoxin concentrations in contaminated oats

In the inoculated oats, HT2-Glc concentrations were mostly higher than HT-2. In the naturally contaminated oats, the concentrations of the sum of T-2 + HT-2, including HT-2 glucoside, were below the maximum limit at all storage conditions except at 0.90 and 0.95 a_w at 20 °C on day 20.

OTA levels were highest at 0.95 a_w for both temperatures and treatments. As a_w rises from 0.90 to 0.95 a_w , OTA increased significantly by 12 % in naturally contaminated oats and by 6% in inoculated oats.

The influence of the interactions of storage conditions on type A trichothecenes and OTA production in contaminated oats are shown in Table 6-7 **Error! Reference source not found.** below.

Table 6-7: The mean values ($n=4 \pm SD$) of the mycotoxin concentrations in the contaminated oats.

Mycotoxins ($\mu\text{g/kg}$)							
Treatment	T($^{\circ}\text{C}$)	Days	a_w	Ochratoxin A	Type A Trichothecenes		
				OTA	HT-2-Glc	HT-2	T-2
Naturally contaminated oats	15	10	0.7	< LOQ*	86.4 ± 41.8^a	68.4 ± 34.6^a	71.3 ± 67.1^a
			0.9	< LOQ*	73.0 ± 24.7^a	134 ± 34.5^b	41.1 ± 22.7^c
			0.95	$17.1 \pm 16.8^*$	67.2 ± 14.5^a	231 ± 154.1^a	16.9 ± 16.8^b
		20	0.7	< LOQ*	61.5 ± 6.5^a	142 ± 92.9^a	62.3 ± 47.3^a
			0.9	$24.5 \pm 31.2^*$	60.0 ± 14.8^a	102 ± 72.7^a	44.9 ± 24.8^a
			0.95	$186.9 \pm 130.8^*$	44.8 ± 19.2^a	130 ± 72.3^b	18.6 ± 19.1^c
	20	10	0.7	< LOQ*	62.7 ± 11.3^a	91.6 ± 54.5^a	66.5 ± 9.1^a
			0.9	$5.4 \pm 4.9^*$	43.8 ± 17.9^a	41.3 ± 25.7^a	27.1 ± 23.5^a
			0.95	$19.0 \pm 19.5^{**}$	47.5 ± 18.4^a	58.0 ± 43.1^a	18.1 ± 16.7^b
		20	0.7	< LOD*	47.9 ± 27.4^a	46.8 ± 21.7^a	223.5 ± 268.6^a
			0.9	$48.2 \pm 65.3^*$	46.1 ± 10.6^a	36.7 ± 11.1^a	1281 ± 1335.4^a
			0.95	$86.2 \pm 66.7^*$	22.8 ± 11.6^a	< LOD ^b	1471 ± 1131.4^a
<i>Fusarium langsethiae</i> inoculated oats	15	10	0.7	< LOD*	78.9 ± 33.1^a	52.4 ± 25.3^a	167 ± 32.8^a
			0.9	< LOQ*	67.4 ± 44.0^a	143 ± 86.0^a	1419 ± 285.1^a
			0.95	< LOD*	178.9 ± 94.2^a	55.3 ± 22.4^b	1564 ± 397.0^a
		20	0.7	< LOD*	103.1 ± 42.8^a	100 ± 50.0^a	135 ± 37.8^a
			0.9	$9.4 \pm 11.8^{**}$	27.6 ± 6.7^a	35.2 ± 7.5^a	2969 ± 658.1^b
			0.95	$115.7 \pm 104.3^{**}$	672.4 ± 384^a	266 ± 102^a	923 ± 315.7^a
	20	10	0.7	< LOD*	112.8 ± 18.1^a	77.6 ± 32.1^a	193 ± 27.5^a
			0.9	< LOD*	32.8 ± 8.9^a	23.3 ± 15.3^a	1568 ± 392.2^a

Chapter 6

	0.95	13.6 ± 12.4*	42.8 ± 25.3 ^a	43.9 ± 15.6 ^a	1283 ± 488.1 ^a
20	0.7	< LOD*	109.7 ± 56.8 ^a	68.9 ± 20.8 ^a	230 ± 121.3 ^a
	0.9	4.1 ± 4.1*	72.1 ± 32.2 ^a	48.2 ± 20.1 ^b	1984 ± 572.4 ^c
	0.95	73.1 ± 95.4*	25.1 ± 14.6 ^a	< LOQ ^b	2866 ± 792.5 ^c

SD- Standard deviation. T-2- T-2 toxin. HT-2- HT-2 toxin. OTA- Ochratoxin A. a_w - water activity. T- Temperature LOD: Limit of detection. LOQ: Limit of quantitation. Concentrations of analytes <LOD and <LOQ are assigned with values of LOD/2 and LOQ/2, respectively. Using non-parametric comparison for each pair (Wilcoxon method), different * shows significant differences in OTA concentrations among a_w for each day and each temperature (within the column). The same letters show no significant differences among the type A trichothecenes concentration at each a_w , each day and each temperature (within each row).

In Table 6-7, T-2 concentrations differed significantly between treatments and storage days. Its concentrations increased as storage days increased and were highest in the inoculated oats.

In both oat treatments at 20 °C, HT-2 concentrations decreased significantly as a_w increased. Additionally, its concentrations significantly decreased as the temperature rose at 0.95 a_w . However, HT-2 concentrations did not vary significantly across all a_w levels at 15 °C in both treatments (4 Table 6-7). HT-2-Glc concentrations decreased insignificantly as the a_w increased in naturally contaminated oats at both temperatures. However, in the inoculated oats, HT-2-Glc concentrations varied significantly at each a_w level at 15 °C. At 20 °C a significant difference in its concentrations was observed at 0.70 a_w . Temperature rise significantly decreased its concentrations only at 0.95 a_w for both treatments (Table 6-7). HT-2-Glc concentrations decreased as temperature and storage days increased and were highest in the inoculated oats.

The concentrations of OTA increased as a_w and storage days increased, with significant differences observed at 0.95 a_w for both days (Table 6-7). OTA decreased significantly in the FLO compared to NO treatment. This is expected due to the *F. langsethiae* inoculum in the inoculated oats.

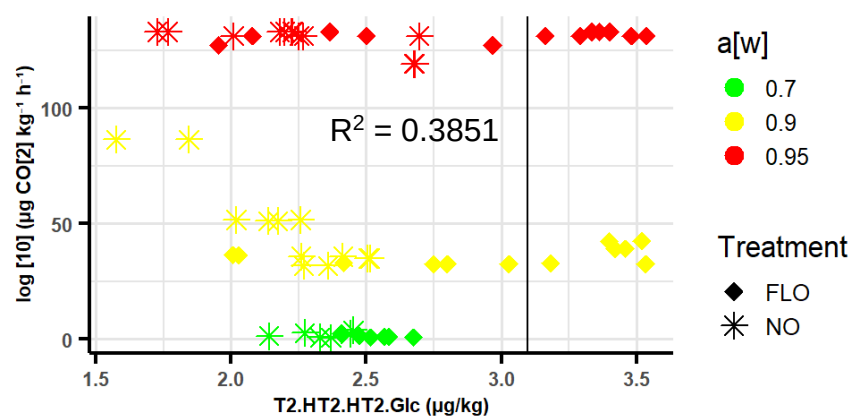
The effects of the interactions of the storage conditions and their p values on type A trichothecenes and OTA concentrations are shown in Table 6-8.

Table 6-8: The effect of the interactions of storage conditions and their p values on type A trichothecenes and ochratoxin A concentrations in contaminated oats

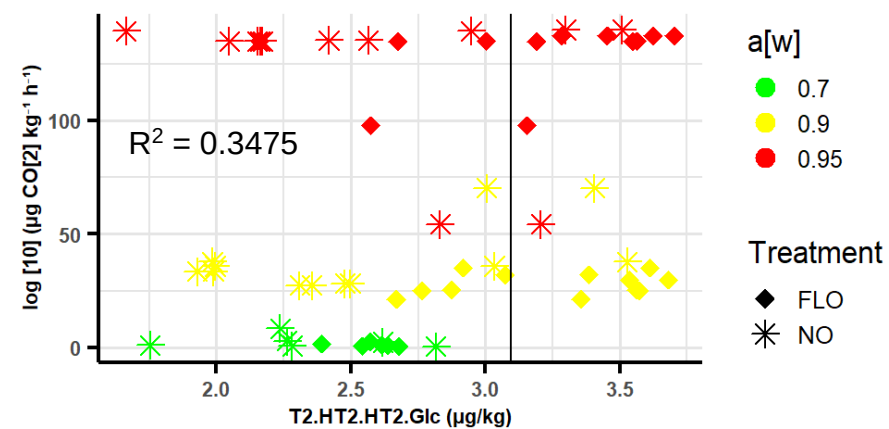
Interaction effects	P values			
	T-2	HT-2	HT-2-Glc	OTA
Treatment	0.000000	0.196070	0.002903	0.001592
a_w	0.193613	0.048085	0.002980	0.000000
Temp	0.157960	0.000000	0.000011	0.740348
Days	0.017844	0.151528	0.210816	0.002197
Treatment*a_w	0.891702	0.831359	0.000353	0.148055
Treatment*Temp	0.156966	0.202035	0.487807	0.559031
a_w*Temp	0.258679	0.000034	0.000000	0.663195
Treatment*Days	0.292155	0.398495	0.053292	0.701857
a_w*Days	0.067817	0.161458	0.618114	0.036380
Temp*Days	0.075635	0.143340	0.689930	0.164303
Treatment*a_w*Temp	0.400962	0.890362	0.000009	0.417415
Treatment*a_w*Days	0.764328	0.914222	0.264151	0.823171
Treatment*Temp*Days	0.146905	0.860472	0.943486	0.533669
a_w*Temp*Days	0.487986	0.000003	0.000553	0.167263
Treatment*a_w*Temp*Days	0.394503	0.010656	0.047801	0.240676

a_w- water activity. Temp- Temperature.

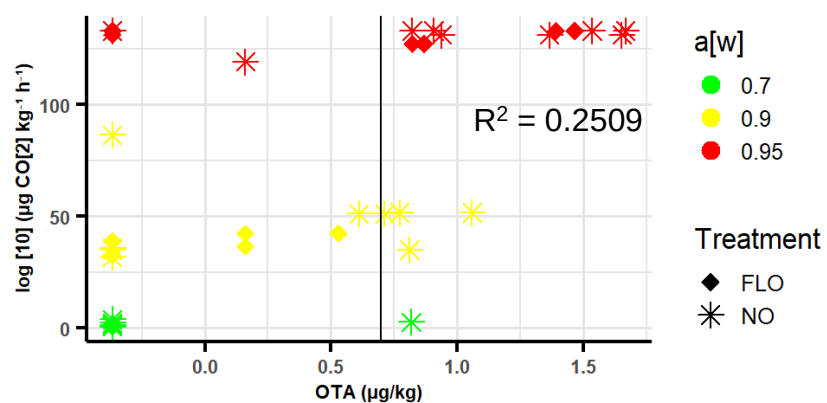
6.3.6 The relationship between respiration rates (RR) and type A trichothecenes and ochratoxin A in oats.



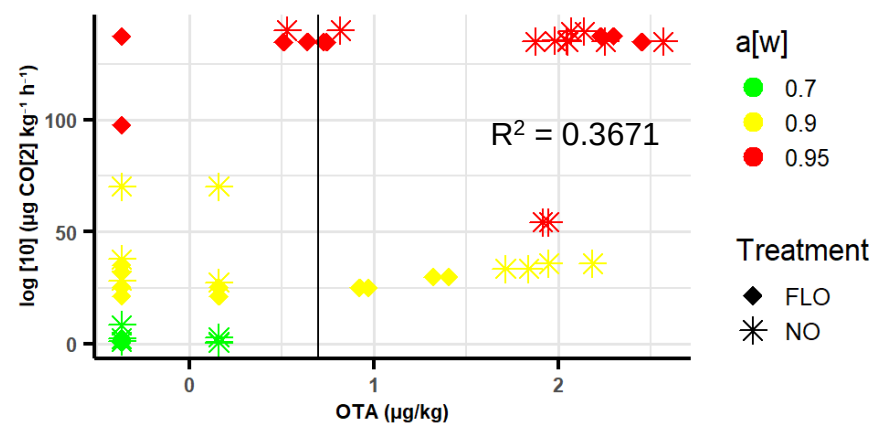
(a) Day 10



(b) Day 20



(c) Day 10



(d) Day 20

Chapter 6

Figure 6-3: The relationship between the respiration rates and the concentrations of (a) the sum of T-2+ HT-2+ HT-2-Glc on day 10 and (b) the sum of T-2+ HT-2+ HT-2-Glc on day 20 (c) OTA on day 10 and (d) OTA on day 20 in the contaminated oats at different water activities. Vertical lines indicate maximum levels of 1250 µg/kg for T-2 + HT-2 and 5 µg/kg for OTA concentrations. NO- naturally contaminated oats FLO- *Fusarium langsethiae* inoculated oats.

Figure 6-3 shows that at 0.70 a_w , RR was low, and the sum of the concentrations of T-2+ HT-2+ HT-2-Glc in the naturally contaminated oats did not exceed the maximum limit. However, as a_w increased, RR increased with a corresponding increase in T-2+ HT-2+ HT-2-Glc concentrations.

On day 10, the sum of T-2+HT2+HT2-Glc exceeded the legislative limit of T-2/HT2 toxin (1250 $\mu\text{g/kg}$) at ≥ 0.90 a_w (22% moisture content) with $\text{RR} > 25$ $\mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ only in the inoculated oats. On day 20, the sum of T-2+HT2+HT2-Glc exceeded the limit at ≥ 0.90 a_w with $\text{RR} \geq 24$ $\mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ in the inoculated oat and at $\text{RR} \geq 37$ $\mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ in the naturally contaminated oats.

Similarly, OTA concentrations increased as CO_2 RR increased and exceeded the maximum limit at ≥ 0.90 a_w , and ≥ 25 $\mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ in the inoculated oats and ≥ 30 $\mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ in the naturally contaminated oats on both days with an exception at 0.70 a_w on day 10 (Figure 6-3c).

The relationship between CO_2 RR, type A trichothecenes and ochratoxins concentrations in the contaminated oats was examined with linear regression and a generalised linear model (GLM). The adjusted R^2 values for each model are shown on the ggplot graphs for each mycotoxin. These values suggest a moderate positive relationship as CO_2 , days and treatment were the significant predictors ($p < 0.05$) of OTA concentrations. A significant correlation was observed between CO_2 and OTA concentrations ($p\text{-value} < 0.0001$) in the contaminated oats with a Pearson correlation coefficient ($r = 0.4705$).

For the T-2+HT2+HT2-Glc model, treatment, a_w and days were the significant predictors. The Pearson correlation coefficient ($r = 0.1159$) shows an insignificant correlation between CO_2 and the sum of T-2+HT2+HT2-Glc ($p\text{-value} = 0.207$). See Table **F-1** in Appendix F.

The model accuracy was assessed through cross-validation by comparing predicted mycotoxin values with known ones (Figure **F-4** and Figure **F-5** in Appendix F), but the linearity of the plots was not satisfactory. These models

alone cannot accurately predict OTA and T-2+HT2+HT2-Glc concentrations using only CO₂ levels as a predictor, as other storage conditions such as a_w , and temperature, influence CO₂ and mycotoxin levels in stored oats.

6.4 Discussion

To the best of our knowledge, the study of the relationship between the real-time CO₂ measurement and the risk of trichothecenes and OTA in oats using a mini-silo pilot study has not been well documented. This study measures for the first time the real-time respiration rates (RR) in stored oats with integrated CO₂ sensors to examine the risk of type A trichothecenes and ochratoxins contamination at storage conditions 0.70, 0.90, 0.95 a_w and 15–20 °C. These a_w levels correspond to the oat moisture content of 14%, 22%, and 25% on a wet-weight basis [35].

Fusarium is commonly known to thrive in wet and humid conditions. Surprisingly in, its populations and isolations at 0.95 a_w were relatively low (see Table 6-2 and Table 6-3). At the same a_w level, *Penicillium* had the highest fungal isolations (100%) at 15°C, indicating that it is a fungus adapted to temperate climates. While *Aspergillus* isolations were highest at 20 °C. Medina and Magan [18] reported the growth limit of *Fusarium langsethiae* strains from the UK and northern Europe on an oat-based media at 0.92–0.93 a_w at 10 °C. No growth was recorded at 0.90 a_w regardless of strain type and temperature studied. A similar study on an oat-based medium, reported no growth rate for the *Fusarium langsethiae* strain at 0.90 a_w , however, growth was recorded at 0.95 a_w at 15 and 20 °C after 10 days of incubation with a growth limit at 0.93 a_w [36]. In contrast to the two studies mentioned, our findings indicate that *Fusarium* can grow *in situ* at a_w levels of 0.70 and 0.90, while showing low or no growth at 0.95 a_w . This may be attributed to the adaptability or resilience of the spores in response to stress from drier conditions [37,38]. This suggests that despite the low overall a_w of the grain, there

may be pockets of wet grains within the silo that can support fungal growth and mycotoxin production.

Respiration rates in contaminated oats

Respiration rates have been an important factor indicating the deterioration of cereal grains in storage. Grains with fungal infections have higher respiration rates because of the increased metabolic activity associated with the infection [39]. Over many decades, research has highlighted that high moisture content leads to high RR in stored grains while grains stored at drier a_w of $0.70a_w$ ($\leq 14\%$ MC equivalent), produced a negligible amount of RR [40–42]. Recent studies have supported this [23,28,43]. Our results show a marked increase in RR as a_w increased in both oat treatments. RR was more than 100% higher at $\geq 0.90 a_w$ for all conditions and treatments. This result aligns with a previous study by Oluwakayode et al. [28] which investigated the impact of a_w and temperature on RR in stored wheat. They reported a rapid increase in RR at a moisture content $> 16\%$ ($0.80a_w$) within the first 10 days of storage in a mini silo of wheat. The elevated level of CO_2 could arise from pockets of grain mass within the silo due to a combination of factors, such as moisture content, the presence of moulds or other microbes, insect infestation, and temperature [22]. Our findings showed that an increase in temperature alone did not significantly increase the RR but its interactions with other storage conditions did. Table 6-5 shows that the two treatments have a significant effect on the respiration rates of the oats, suggesting an inoculum effect on RR. An important observation is that the CO_2 produced in both oat treatments was highest at $0.95 a_w$ however, it did not correspond to *Fusarium* growth at the same a_w level. The CO_2 at each a_w was produced by both the grain and the fungal communities which include both spoilage and toxigenic moulds within the oats in the silo. It is therefore unrealistic to separate the respiration of the grain from the respiration of the fungus during CO_2 monitoring. As CO_2 increased and become saturated within the silo, the CO_2 sensor's concentration limit (50,000 ppm) was reached, and the readings remained constant throughout the storage. Hence, in both oat treatments, the

wettest water activity (0.95 a_w) showed the same real-time respiration rates from day 10 until day 20

Type A trichothecenes in contaminated oats

Despite observing low or no *Fusarium* growth from the fungal population and isolation results at 0.95 a_w for both treatments, considerable levels of type A trichothecenes were found in the oats samples with T-2 concentrations alone exceeding the maximum limit of the sum of T-2+ HT-2 (1250 $\mu\text{g/kg}$) in most cases. It is understood that *Fusarium*'s growth may be suppressed under low-oxygen and high-humidity conditions prompting it to produce mycotoxins such as fumonisins, zearalenone, and trichothecenes to deter microbial competition and adapt to stress [44]. *Fusarium* regulates growth and toxin synthesis through separate metabolic pathways, allowing toxin production independent of growth [45].

In the naturally contaminated oats, T-2 toxin levels decreased by 1.5 times as a_w increased. However, on day 20 at 20 °C, T-2 concentrations were 6 times higher as a_w increased from 0.70 a_w to 0.90 a_w and a 48% increase from 0.90 to 0.95 a_w . Under low and medium conditions, the concentrations of HT-2-Glc were higher than those of T-2. However, in the wettest conditions with high levels of T-2, this pattern reversed. This suggests that the plants may have been overwhelmed by the toxin, preventing them from detoxifying it effectively. The significant levels of HT-2-Glc highlight the necessity of measuring it to ensure accurate assessments of the total mycotoxin content in grains.

Additionally, HT-2 concentrations were more than two times significantly ($p < 0.05$) higher than T-2 concentrations at wet conditions (0.90–0.95 a_w) at 15 °C. However, in the inoculated oats, T-2 concentrations were more than 2.7 times higher than HT-2 concentrations. This may be due to inoculum load in the inoculated oats. The 0.70 a_w grains were not inoculated but used as a control experiment.

Some studies have reported higher levels of HT-2 than T-2 toxins in oats although with no consideration of the impact of different environmental factors [46–48]. The higher concentrations of HT-2 than T-2 could be due to the deacetylation of T-2 to HT-2 by oat grains or the rapid hydrolysis of T-2 to HT-2 by the natural mycobiota in response to ecological stress. Further investigation is needed to understand the metabolic pathways of HT-2 in *F. langsethiae* [49].

In naturally contaminated oats, it is expected that the concentrations of type A trichothecenes will increase under wet conditions, as most *Fusarium* species thrive in such environments. However, *Fusarium langsethiae* has been noted to have slow colonization rates, having an inability to compete with other phyllosphere *Fusarium* species, such as *F. poae*, in colonizing oats [10,50]. Kahla et al. [51] reported variations in the effect of the acclimatisation of a range of *F. langsethiae* strains on growth rates and toxin production under ambient (400 ppm) and elevated (1000 ppm) CO₂ on oat-based media. Further research is necessary to understand the interactions between *F. langsethiae* and other mycobiota, as well as the implications of such competition for dominance in the oat phyllosphere and its impact on toxin production.

OTA in contaminated oats

Ochratoxin A is generally considered a storage toxin produced mostly by *Penicillium* or *Aspergillus* species. It was interesting to observe a measurable amount of OTA produced in the stored oat alongside *Fusarium* toxins. Hence, OTA concentrations were reported in this chapter. OTA increased with temperature rise except at 0.95 a_w on day 20 in the naturally contaminated oats while in the inoculated oats, OTA decreased with temperature rise at both 0.90 and 0.95 a_w. The highest OTA level was at 15 °C, and 0.95 a_w exceeding the maximum limit (5 µg/kg). This can be linked to the high prevalence of *Penicillium* populations or isolations in the oats at 0.95 a_w and 15 °C where *Fusarium* populations were least observed. However, the concentration of OTA was lower than that of type A trichothecenes under these storage conditions. A similar study reported OTA production to be highest at 0.95 a_w and 15°C in stored

wheat grains [28] supporting that *Penicillium* thrives well in temperate conditions. Cairns-Fuller et al. [52] reported that *P. verrucosum*'s growth and OTA production can occur at $\geq 0.85 a_w$ in wheat grains. Our findings observed measurable values of OTA $\geq 0.90 a_w$. Other studies have reported high OTA levels at $\geq 0.90 a_w$ [53–55].

It is important to stress that the effect of the interaction of a_w x storage days significantly increased OTA concentrations in the stored oats. Similar to our findings, Dhungana et al [55] reported a significant impact of the interaction of a_w x incubation period on OTA accumulation. The highest OTA level was at $0.90 a_w$ and it increased with a prolonged incubation period.

Relationship between CO₂ and mycotoxins in contaminated oats

Inoculation was performed to increase the prevalence of *F. langsethiae* in the natural mycobiota of the oats to assess its impact on the concentrations of type A trichothecenes in the event of a future population increase due to silo infiltration or climate changes. Table 6-7 **Error! Reference source not found.** shows that more T-2+HT-2+HT-2-Glc concentrations exceeded the maximum limit (1250 $\mu\text{g/kg}$) at $\geq 0.90 a_w$ for inoculated oats than in the non-inoculated treatment. OTA concentrations were also lower in the inoculated oats suggesting the prevalence of *F. langsethiae* over *Penicillium* species.

The concentrations of the sum of type A trichothecenes and OTA were not strongly correlated with the RR produced in the stored oats. We hypothesise that the RR was produced by the entire fungal community and the oats grains, not only by *Fusarium* and *Penicillium* species. Also, a direct correlation of RR with these mycotoxins may not be feasible as other storage conditions influence changes in the RR and the mycotoxins produced. However, our findings suggest that CO₂ monitoring is a good indicator of fungal activity and mycotoxin contamination in stored oats. Therefore, at $\geq 0.90 a_w$ and RR of $\geq 25 \mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$, the sum of type A trichothecenes and ochratoxin A will exceed their

legislative maximum limits in stored oats. Monitoring environmental factors during oat storage is important to minimise mould growth and mycotoxin contamination.

6.5 Conclusion

This study shows the behaviour of *F. langsethiae* within a natural mycobiota in stored oats, producing high levels of T-2 toxins at the wettest condition of 0.95 a_w despite appearing to be outgrown by *Penicillium* species. The water activity of grains and the storage days (duration) significantly affected RR and the concentrations of type A trichothecenes and OTA. The linear regression model shows a moderate positive relationship between CO₂ RR and OTA concentrations. Mycotoxin exceeded legislative limits at $\geq 25 \mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ at $\geq 0.90 a_w$ in the naturally contaminated oats. These findings established the potential of CO₂ RR as an indication of the relative risks of oats colonisation in storage by these mycotoxins and its importance as part of an integrated approach for developing decision support systems to improve oats storage management.

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7 Cost-benefit analysis of the integrated CO₂ sensing approach as a decision support system (DSS) tool

Abstract

The post-harvest losses of grains in storage have been a major concern for many years. Research suggests an integrated sensing approach can help minimise grain spoilage by reducing mould growth and mycotoxin contamination in stored grains. This knowledge will be used to develop a decision support system (DSS) tool to improve grain management. This chapter used a cost-benefit analysis to illustrate the costs and savings associated with post-harvest handling of grains by comparing four different DSS approaches. This approach consists of grain silos having (i) no sensor (ii) temperature (T) sensor (iii) relative humidity (RH) + T sensors and (iv) carbon dioxide (CO₂) + RH+ T integrated sensors. The latter approach has been suggested as a more sustainable option for long-term grain management.

7.1 Introduction

Cost-benefit analysis (CBA) is a data-driven approach used to evaluate the financial benefits and costs of a project or decision from a business perspective [1]. It involves calculating all the costs associated with a project or decision and subtracting that amount from the projected benefits. This method helps identify hidden costs and benefits, making decision-making easier. However, predicting all the factors that could impact the outcome of a decision can be challenging. Unforeseen changes in market demand, material costs, and the global business environment, particularly in the long term, can be unpredictable [2]

Previous chapters in this thesis have highlighted the importance of mitigating the risks of mould growth and mycotoxins in cereal grains due to their detrimental health and economic effects. An integrated approach (CO₂, temperature, and relative humidity sensors) has been suggested to be critical in developing a DSS

for improving grain storage management. However, it is crucial to assess the costs and benefits of this DSS tool to determine its affordability/acceptability by grain farmers and managers to ensure its sustainability within the food chain system.

This CBA aims to assess various integrated sensing systems as DSS tools and address important questions, such as whether these DSS tools are worth the investment and what the relevant limitations of using them are. This technique is fundamental to making informed decisions regarding the safety and quality of stored grains for food and feed use.

7.2 Methodology and Experimental Design

The cost-benefit assessment of the DSS tools was analysed in a Microsoft spreadsheet. The scope of the study was to compare the cost-effectiveness of different DSS tools to help farmers choose the most effective tool for grain storage improvement.

The Experimental design

The cost incurred by the farmer during cultivation, preharvest, transportation to storage facilities and storage equipment were all excluded. Investment cost focused only on the purchase and installation of DSS tools, where appropriate. The purchase cost for the DSS tool was obtained from the Gescaser company (Gescaser, Lleida, Spain). Assumptions were made for the operational costs/expenses. The estimated price of wheat per tonne in November 2024 was £184 according to AHDB [3]. The estimated losses were associated with storage only. While there are no current specific percentage losses associated with each DSS tool in the UK, 5% loss or higher was reported as grain rejection associated with storage problems [4]. This was used to estimate the percentage losses for each silo.

7.2.1 The cost analysis – case study

A grain storage site having four silos received 45 tonnes of 2024 Autumn harvested wheat. Each silos have zero, one, two, and three sensors. The cost and benefits comparisons for the four silos of wheat grains stored over five years were analysed. This will help the farmer/grain manager to decide which DSS tool is suitable for grain safety and profitability for long-term storage. The silos model is 9.1 m diameter, 12.2 m. eaves height and 14.83 m. total height. The DSS tool parameters are in Figure 1.

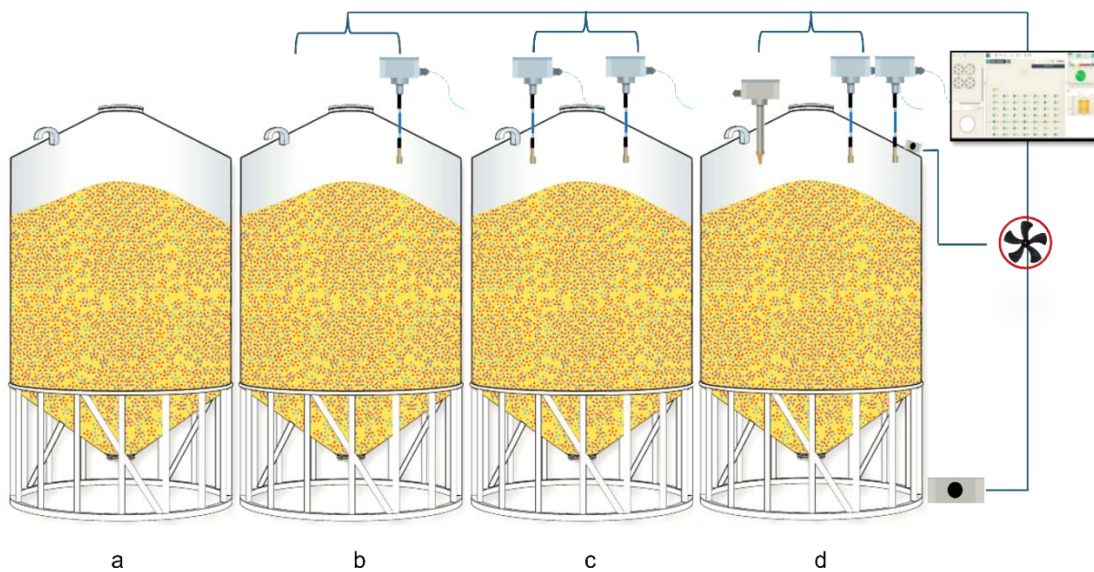


Figure 7-1: Grain silos with different decision support system tools.

(a) No sensor (b) Temperature sensor (c) Temperature and relative humidity sensors (d) CO₂, temperature and relative humidity sensors.

7.2.2 The integrated sensor system

An integrated sensor system monitors in real-time, CO₂, temperature, and RH in grain silos. These sensors acquire real-time data through a simple-to-install design that can be integrated into silos of the food and feed processing industries (more information on the sensors can be found at: www.gescaser.com). Data collected by these sensors are linked to previously input models in the DSS for real-time prediction of mould growth and the presence/absence of the specific

mycotoxin(s) in the silos. The sensors are designed to be ATEX compliant i.e. to avoid the risk of sensors catching fire.

According to van Der Fels-Klerx et al. [5], each sensor system component includes the following elements:

- ❖ Power source.

The system's power source was designed to minimise total power consumption by switching between monitoring with each sensor in sequence, thus minimising the heat generated during the measurements. The electronic design was also integrated with a wireless module that allowed the transmission of the measured signals to a remote-control module either wirelessly or physically cabled.

- ❖ Multiplexer board.

The multiplexer board was used to manage and switch between the sensor nodes in sequence providing the data on actual CO₂, temperature and RH.

- ❖ Nodes.

Each cable contained a string of four sensor nodes with each node containing the integrated CO₂, temperature and RH sensors. The quantity of nodes per cable is limited by the total power consumption.

- ❖ A computer system

The computer system receives signals from the sensors onto a designed software. It combines it with other data related to the silos, including data related to the structure of the installation (number of silos, number of lines, number of sensors) and the grain type stored. These data are used to run the prediction models; the outcomes being predictions of the fungal/toxin status of the grain in the silo, are displayed to the end-user.

7.2.3 The cost analysis for each integrated sensing approach

1. Initial investment:

- Cost of CO₂ integrated sensors: The initial cost for purchasing the CO₂ + RH +T sensors (Gescaser, Lleida, Spain) required for monitoring is £5,637. The T sensor and the RH +T sensors cost £2500, and £3250 respectively. All costs are in Pounds and the exchange rate from euros to pounds for the CO₂ + RH +T sensor price was taken as €1= £0.87

2. Installation and calibration:

- Installation costs: Installation may require professional assistance, particularly for larger storage facilities. Labour costs were considered for the national minimum wage of £11.44 in 2024 [6].
- Calibration expenses: Regular calibration of the sensors is necessary to ensure accurate measurements. Calibration kits and technician fees contribute to ongoing expenses.

3. Maintenance and upkeep:

- Sensor maintenance: Regular maintenance, including cleaning and sensor replacement, is essential for optimal performance.
- Electricity cost: The cost of powering the sensors in the silos.
- Data management: Costs associated with data collection, analysis software, and personnel for data interpretation and decision-making.

7.2.4 The benefits analysis:

1. Early detection of mould growth:

- Timely warning: CO₂ integrated sensors provide early detection of increased CO₂ levels, indicating the onset of mould growth before visible signs appear.
- Preventive measures: early detection allows for prompt intervention, such as adjusting temperature and humidity levels, aeration or applying appropriate treatments, to mitigate mould proliferation.

2. Reduction of mycotoxin contamination:

- Preventing mycotoxin formation: by detecting mould growth early, CO₂ integrated sensors help prevent the production of mycotoxins, reducing the risk of contamination in stored grains.
- Compliance with regulations: implementing effective monitoring systems can help meet regulatory requirements and maintain food safety standards, avoiding potential penalties and recalls.

3. Preservation of grain quality:

- Preservation of nutritional value: minimising mould contamination preserves the nutritional quality of grains, ensuring their suitability for human and animal consumption.
- Preservation of market value: maintaining high-quality grains enhances marketability and prevents financial losses associated with downgraded or rejected products.

4. Cost savings:

- Reduced losses: early detection and intervention minimise losses due to mould-infested grains, preserving inventory and minimising financial losses for farmers and distributors.
- Lower treatment costs: timely interventions may reduce the need for costly remediation measures, such as fumigation or disposal of contaminated batches.

Table 7-1 below shows the estimated costs of expenses incurred/ year over 5 years for one silo of grain. The annual staff cost for the sensed silos was £11.44/hr for 2 hours of data evaluation twice a week. In the No sensor, we assumed that a staff is employed to turnover the grains for 2 hours a week at the minimum wage of £11.44. The annual cost was subtracted from the annual income.

Table 7-1: Estimated expenses for each integrated sensing approach in grain silos for five years.

	T sensor					RH + T sensors					CO ₂ + RH +T sensors				
	Year 1	Year 2	Year 3	Year 4	Year 5	Year 1	Year 2	Year 3	Year 4	Year 5	Year 1	Year 2	Year 3	Year 4	Year 5
Software maintenance/renewal	£0	£0	£100	£0	£0	£0	£0	£100	£0	£0	£0	£0	£100	£0	£0
Calibration expenses	£0	£40	£40	£40	£40	£80	£80	£80	£80	£80	£0	£120	£120	£120	£120
Electricity & Internet	£100	£100	£100	£100	£100	£150	£150	£150	£150	£150	£200	£200	£200	£200	£200
Sensors repair and maintenance	£0	£100	£150	£0	£100	£0	£100	£250	£0	£100	£0	£100	£300	£0	£100
Staff cost/ data management	£1,098	£1,098	£1,098	£1,098	£1,098	£1,098	£1,098	£1,098	£1,098	£1,098	£1,098	£1,098	£1,098	£1,098	£1,098
Total Expenses	£1,198	£1,338	£1,488	£1,238	£1,338	£1,328	£1,428	£1,678	£1,328	£1,428	£1,298	£1,518	£1,818	£1,418	£1,518

T- Temperature. RH- Relative Humidity. CO₂- Carbon-dioxide.

Table 7-2: The cost-benefit analysis of a wheat silo with/without integrated sensors for five years.

Cost - Benefit	No Sensor	T	RH+T	CO ₂ +RH+T
Tons of grains in a 9.1m diameter silo 45 tonnes.	45	45	45	45
C1: Investment cost: Sensors set up	0	£2,500	£3,250	£5,637
C2: Sensor's installation cost	0	£100	£150	£200
C3: Monitoring Computer cost	0	£450	£450	£450
The sum of Investment costs (C1+ C2+ C3)	0	£3,050	£3,850	£6,287
Percentage loss of grains in silos	45%	30%	25%	5%
Loss of grains from 45 tonnes	20.25	13.5	11.25	2.25
New tons of grains in the silo after percentage loss was deducted	24.75	31.5	33.75	42.75
Income after %loss (price/ton Nov 2024= £184)	£4,554	£5,796	£6,210	£7,866
Year 1 Expenses + sum of investment cost	£1,098	£4,248	£5,178	£7,585
Year 1 Profit/loss= Income-Investment	£3,456	£1,548	£1,032	£281
Due to grain quality loss in the No Sensor or Temperature silos, grains may be downgraded to feed with lower prices in the following years				
Year 2 Price /ton cost (£)	140	140	184	184
Year 2 Income= New price of grains silo sold	£3,465	£4,410	£6,210	£7,866
Year 2 Profit/loss= Income- expenses	£2,367	£3,072	£4,782	£6,348
Year 3 Price /ton cost (£)	£140	£140	£184	£184
Year 3 Income= New price of a silo of grains sold	£3,465	£4,410	£6,210	£7,866
Year 3 Profit/loss= Income- expenses	£2,367	£2,922	£4,532	£6,048
Year 4 Price /ton cost (£)	£140	£140	£184	£184
Year 4 Income= New price of a silo of grains sold	£3,465	£4,410	£6,210	£7,866
Year 4 Profit/loss= Income- expenses	£2,367	£3,172	£4,882	£6,448
Year 5 Price /ton cost (£)	£140	£140	£184	£184
Year 5 Income= New price of a silo of grains sold	£3,465	£4,410	£6,210	£7,866
Year 5 Profit/loss= Income- expenses	£2,367	£3,072	£4,782	£6,348
Total profit/loss	£12,924	£13,786	£20,010	£25,473

T- Temperature. RH- Relative Humidity. CO₂- Carbon-dioxide.C1,2,3 – Cost 1,2,3.

In Table 7-2 for the No Sensor silo, the expenses deducted from the income from year 2 to year 5 were the staff costs (£1,093), as no monitoring was in place. Meanwhile, for the other three silos, the total expenses incurred in each year (Table 7-1) were deducted from their respective income.

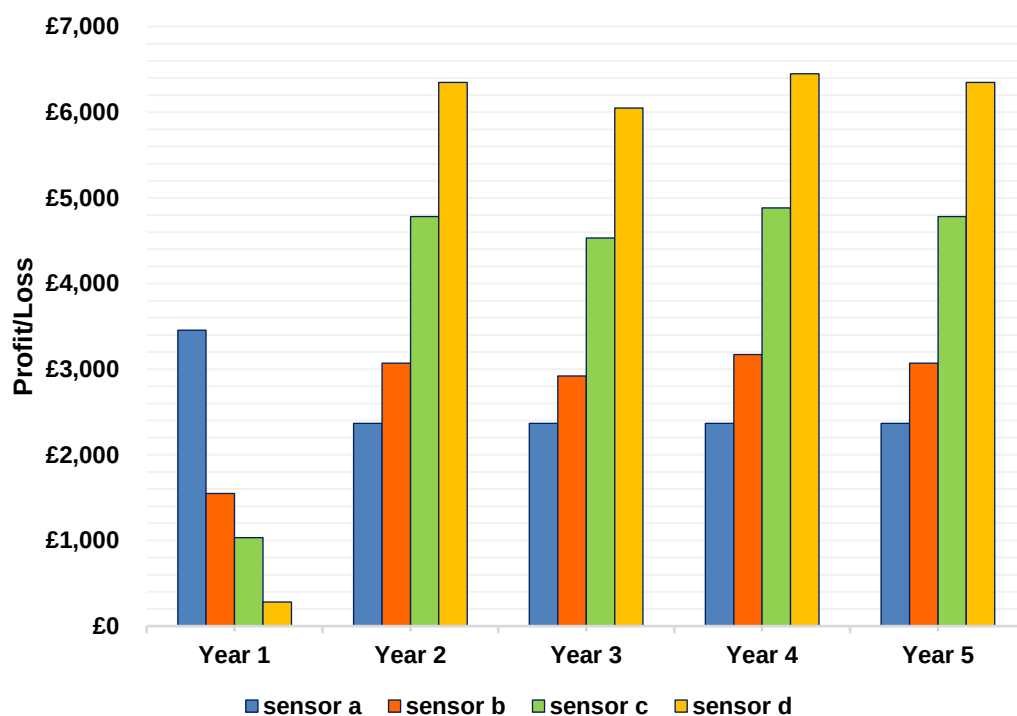


Figure 7-2: The profit/ loss for different integrated sensing approaches over five years. T: temperature. RH: relative humidity. CO₂: Carbon dioxide.

In the first year, the 'No Sensor' silo was the most profitable among the four silos. The CO₂+RH+T sensors benefit was 12 times lower than the No Sensor and was 3 times lower than the RH+T sensors. However, its benefit peaked in the second year and was consistent in subsequent years. In year 5, the benefits of the CO₂+RH+T sensors were more than 2 times higher than the No Sensor and the Temperature sensor, while it was 1.3 times higher than the RH+T sensors (Figure 7-2).

The low profit for the CO₂+RH+T silo in year one was attributed to the investment cost in purchasing the integrated sensors which only happens at once, after which there was a return on investment as the sale of wheat will mostly be at standard prices with minimal postharvest loss. This indicates that the benefit of the CO₂+RH+T sensors is sustainable for long-term storage. Should there be

changes in wheat prices in the future, the possibility of grains downgrading to feed with a price drop is limited since the DSS tool ensures the safety of grains.

The RH+T sensors appear to be more profitable than the No Sensor silo and the temperature sensors, but their percentage grain loss could increase and raise concerns over long-term storage. The temperature sensor was only 1.3 times better than the No Sensor silo from year 2- year 5. Grains in the Temperature and No Sensor silos are prone to rapid spoilage, especially with increasing climate conditions which are favourable to mould growth and mycotoxin production in grain silos [7-9].

The total profit/loss represents the total net present value (NPV) and was calculated by subtracting the total present value of costs from the total present value of benefits.

Table 7-2 above shows that the integrated CO₂+RH+T sensor's CBA had a negative net present value (NPV) for the first year. However, the NPV was positive from the second year and had the highest total NPV among all three sensor types. A positive NPV shows that the benefit of the CO₂+RH+T sensor outweighs the cost, indicating that it is economically viable. It is important to highlight that a farmer/ grain manager would receive the return on investment made on the integrated sensing approach from the second year, as the profit made in year 2 is £6,348, which is more than the investment and set-up costs of £6,287.

In years with adverse climatic conditions, the CO₂+RH +T sensors can offer an early warning of potential grain spoilage. This timely indication enables farmers or grain managers to take prompt action, such as employing grain ventilation or drying and aeration techniques or releasing grains for further processing to safeguard against safety/quality deterioration. Consequently, this approach helps minimise waste and enhance profitability.

7.3 Conclusion

Implementing the integrated CO₂+RH+T sensors for monitoring mould growth and mycotoxin contamination in stored grains offers significant benefits in terms of early detection, prevention of mycotoxin contamination, and preservation of grain quality. While initial investment and ongoing maintenance incur costs, the potential for reducing losses and ensuring food safety outweighs these expenses.

The experiments in previous chapters have shown that the CO₂ sensors as part of an integrated system with temperature and relative humidity sensors are an effective indicator of mould growth and mycotoxin contamination in wheat and oats grains silos. Therefore, they are a valuable DSS tool for improving grain storage management.

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8 General discussion, conclusions and recommendations

8.1 The initial mycobiota of UK cereal grains

Cereal grains are of great agricultural significance in the United Kingdom (UK) providing food, feed, and fuel. The UK produces over 100% of domestic consumption of oats and barley and over 90% of wheat grains [1]. However, due to the UK's temperate climatic conditions, these grains are more prone to fungal attack and mycotoxin contamination. Increasing seasonal changes will further exacerbate this issue [2,3]. **Chapter two** shows that 45% of the grains analysed had water activity above the safe threshold risk of 0.70 a_w . Grains from England and NI were mostly contaminated with *Alternaria*, *Aspergillus* section *Aspergillus* (formerly called *Eurotium*), *Penicillium*, *Fusarium*, *Cladosporium*, *Aspergillus* and *Mucor* genera. *Penicillium* had the highest frequency of isolation among the potentially mycotoxigenic fungal genera. However, the ultraviolet screening shows that the *Penicillium* isolated from the grains were not mycotoxigenic. *Fusarium* toxins were found in all grains except in Maize from the Northern Ireland 2020 harvest which was mostly dominated by *Aureobasidium* species. This highlights the UK's wet weather conditions, which provide an ideal environment for *Fusarium* to thrive [4,5].

8.2 Impact of storage conditions on mycotoxins in oats and wheat grains

Moisture content (MC) and temperature are two important abiotic factors that influence fungal growth and the mycotoxin contamination of grains [6]. It is therefore important that grains are subjected to efficient drying regimes mostly below 14% MC or 0.70 a_w before storage to minimise mould growth and mycotoxin contamination. Storage conditions can be comprised either through a rise in temperature, rainfall, insufficient drying of grains after harvest, or water ingress into silos through silo leaks or the presence of insects and pest activity [7].

Previous research has considered the concentrations of regulated mycotoxins under different storage conditions. Nonetheless, **chapters three and four** described for the first time how the different environmental factors influence the ratios of concentrations of regulated and conjugated mycotoxins including emerging mycotoxins in wheat and oat grains. Grains were irradiated to reduce microbial load while retaining their germinative capacity [8]. Irradiated grains were inoculated to examine the behaviour of the fungi for growth and mycotoxin production outside its natural mycobiota.

Fusarium langsethiae growth was pronounced in the irradiated grains and evidenced by the high concentrations of type A trichothecenes. However, in naturally contaminated grains, the sum of T2, HT-2, and HT-2-Glucosides was lower than the sum of DON and DON-3-G suggesting that the growth of other *Fusarium* species might outcompete *Fusarium langsethiae* [9].

In some cases, for example in **chapter three**, at the wettest a_w level, the concentrations of DON's conjugated toxin (DON-3-G) were high enough to increase the total DON content in the oats, exceeding the UK's legislative limits (1750 $\mu\text{g/kg}$) [10].

Similarly, in the naturally contaminated wheat grains in **chapter four**, the concentrations of DON-3-G were higher than DON levels at 0.93 a_w while ZEN-14-S was higher than its precursor ZEN at 0.98 a_w at both temperatures (20–25 °C).

Mycotoxin levels in the inoculated grains indicate a high risk of contamination. This risk could occur due to inefficient drying regimes or changes in the storage environment, especially if the populations of *Fusarium graminearum* or other *Fusarium* species are high in grains. These findings highlight the importance of conducting a robust mycotoxin analysis in grains to avoid underestimating the total mycotoxin content of grains.

8.3 CO₂ production in grain mini silos as an indicator of fungal activity and mycotoxin contamination

Grains and moulds respire, producing CO₂ in storage. However, if storage conditions such as moisture and temperature are not properly managed, grain safety and quality can quickly deteriorate [6]. Mycotoxins can spread from a hotspot area to the entire grain mass in the silo. To monitor this, CO₂ sensors integrated with relative humidity (RH) and temperature sensors were used to measure respiration rates (RR) in grain silos. Previous studies have measured CO₂ production in stored wheat, maize or peanuts by injecting CO₂ from an accumulated headspace of the stored commodity into the gas chromatography (GC) [11–14]. However, there could be biased readings in CO₂ measurements if the sampled volume does not accurately represent the entire headspace. Also, there could be minor air leaks or diffusion during syringe withdrawal which might contaminate the sample [15].

Other studies have monitored CO₂ production in stored wheat and maize with either separate or integrated sensors [16–19]. However, only Deoxynivalenol (DON) was analysed from contaminated stored wheat using the enzyme-linked immunosorbent assay (ELISA) method [18]. Also, the grain sizes used in these studies were smaller compared to those used in this research.

This research (in **chapters five and six**) monitors for the first time the real-time CO₂ RR in mini silos of wheat and oats using CO₂ sensors integrated with relative humidity (RH) and temperature sensors to examine if the RR produced can predict Ochratoxin A and the sum of type A trichothecenes contamination exceeding legislative limits. Their conjugated mycotoxins were also analysed with Liquid chromatography-tandem mass spectrometry (LC-MS/MS).

A linear regression model was used to analyse the RR and the mycotoxins data set for prediction. **Chapter five** shows that OTA – a nephrotoxin and an important storage toxin (especially in temperate regions) mostly produced by *Penicillium* and *Aspergillus* species had a positive correlation with CO₂ RR at wet conditions and exceeded maximum limits at ≥ 0.90 a_w (22% moisture content) and ≥ 15 µg CO₂ kg⁻¹ h⁻¹ in a mini-silo of wheat.

Similarly, **chapter six** highlighted that OTA and T-2 + HT-2 toxin contamination exceeded legislative limits at $\geq 0.90 a_w$ (22% moisture content) and $\geq 25 \mu\text{g CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ respectively at $>0.80 a_w$ in the stored naturally contaminated oats.

These findings established the potential of CO_2 RR as an indication of the relative risks of grain colonisation by these mycotoxins and its importance as part of an integrated approach to developing decision support systems to improve grain storage management.

Another important observation is that the fungal populations and isolations of the potentially mycotoxigenic fungal genera and the mycotoxins profile of the contaminated grains from the storage experiments in **chapters 5 and 6** at wet conditions differed from those from the initial mycobiota of the grains in chapter 2 due to the interactions of the different storage conditions. For example, in **chapter 2**, the initial value of DON-3-G was higher than DON in wheat at $0.70 a_w$, it maintained this trend at $0.93 a_w$, however at $0.98 a_w$ its concentrations were lower than DON's concentrations in **chapter 4**.

8.4 Could the CO_2 DSS system be cost-effective?

The experiments in previous chapters (5 and 6) have shown that CO_2 sensors as part of an integrated system with temperature and relative humidity sensors are effective indicators of mould growth and mycotoxin contamination in wheat and oats grains silos. **Chapter 7** compares the costs and the benefits of the CO_2 integrated systems as a decision support systems (DSS) tool to other DSS tools without the CO_2 sensors. Although the initial cost of setting up the CO_2 DSS was higher than others, the benefits outweighed the costs for long-term storage. Implementing the decision support tool of $\text{CO}_2 + \text{RH} + \text{T}$ sensors in grain silos offers significant benefits in the early detection and prevention of mycotoxin contamination and the preservation of grain safety and quality thereby reducing losses, ensuring food safety and increasing profitability for grain managers. Therefore, they are economically valuable tools for improving grain storage management.

8.5 Conclusions and contributions to knowledge

All contributions to knowledge stress the importance of efficient drying and monitoring of grains before and during storage by grain farmers/traders.

The use of CO₂ sensors will alert farmers to release grains in silos for further drying/ processing to reduce food waste and food costs, thereby increasing the safety of grains for consumption.

This research also informs policymakers about the necessity of considering conjugated mycotoxins and other secondary metabolites, as current legislation focuses only on regulated mycotoxins. It encourages them to develop policies to detect, monitor, and mitigate mycotoxins, ensuring food safety to enhance the health of both humans and animals and to improve economic stability.

The following are the contributions to scientific knowledge

- The fungal diversities focusing on the potentially mycotoxigenic fungal genera of grains from the UK have been established.
- This work has shown for the first time that different abiotic factors affect other fungal secondary metabolites and the ratios of the concentrations of regulated and conjugated mycotoxins in stored wheat and oats.
- This work shows for the first time that real-time CO₂ respiration rate in mini silos of grains stored at different abiotic factors (water activity x temperature) predict the relative risk of OTA contamination in stored wheat and oats. Also, the storage conditions where OTA contamination exceeded the legislative limit were identified.
- It is important to highlight that while there is a relationship between CO₂ and OTA contamination, they are influenced by other storage conditions.

Overall conclusion

The integrated management of mould spoilage risks in stored grain entails key strategies as discussed in this work for preventing mould development by maintaining grain moisture levels below the critical threshold for fungal growth. Accurate monitoring of grain a_w and temperature fluctuations during the storage period, while also tracking early indicators of respiration activity of storage fungi and implementing physical interventions to reduce moisture accumulation within the grain bulk.

These strategies, in addition to physical treatments or biocontrol, will collectively aim to effectively manage and minimise the risk of mould spoilage in stored grain.

8.6 Recommendations for future research

Post-harvest management of grains requires a holistic approach involving, grain managers/industries, researchers and policymakers.

Here are key research aims for future experiments derived from this thesis, highlighting potential avenues for further study.

- Grains from other locations, such as Scotland and Wales in the UK can be analysed for fungal diversities to give a comprehensive understanding of the mycobiota of UK grains.
- Polymerase Chain Reaction (PCR) analysis with gel electrophoresis can be done to identify the 'species' of the potentially mycotoxigenic strains to know the fungal spectrum contaminating UK grains
- CO₂ sensing should be conducted on field experiments (farm/ commercial silos) to understand how fieldconditions directly influence real-time respiration and mycotoxin contamination in grains.
- The reading capacity of the CO₂ integrated sensors should be increased (> 50100 ppm) to allow more real-time respiration measurements from the grain silos over the long storage period.

Chapter 8

- Development of immunosensors that capture mycotoxin conjugates to monitor the total mycotoxin content in grains for trade/exportation purposes.
- The use of the generated data and knowledge in this research in addition to other modules to build a complete DSS to improve grain management.

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Appendices

Appendix A Ethical approval letter

Part 1: Summary Details

Lead Researcher (Applicant)

Title	First Name	Surname
<input type="text" value="Mrs"/>	<input type="text" value="Abimbola"/>	<input type="text" value="Oluwakayode"/>
School	<input type="text" value="School of Water, Energy and Environment"/>	
Course code	<input type="text" value="DPAGIFRC"/>	
Email	<input type="text" value="Abimbola.Oluwakayode@cranfield.ac.uk"/>	
		☐ Student ☐ Staff

Please confirm mode of study

☒ Full-time

Please confirm method of study

☐ Taught (MSc, MBA)

☐ Research (PhD, EngD, DBA, MSc by Research)

Please confirm which project type your application is supporting

☐ Thesis

☐ Group Project

☐ Other

Does this research project involve any co-researchers?

Appendices

Yes

No

Short title of project

Development of a novel and effective post-harvest decision support system (DSS) for stored cereals to minimise mould spoilage and mycotoxins in food

Full title of project

Development of a novel and effective post-harvest decision support system (DSS) for stored cereals to minimise mould spoilage and mycotoxins in food

Abstract (In approximately 300 words, describe your proposed project including research methods, identification of potential risks and measures to reduce these risks)

: UK grain production represents a value of £2 Billion in ex-farm gate value. Post-harvest losses and rejection due to fungal spoilage and mycotoxin contamination often represents between 5-10% of this value, especially in wet harvest years. This has significant impacts on food/feed chains where rejection has occurred. It is thus critical that during grain drying and post-harvest storage, nutritional quality is conserved for down-stream processing. This is important economically for farmers and for the grain trade and processing industries. The problem is that while there is a significant focus on pre-harvest disease control, post-harvest losses due to fungal spoilage and mycotoxins have received less attention although they can result in rejection and thus significant economic losses. This PhD project is thus going to contribute in the development of a rapid and reliable Decision Support Systems (DSS). This could be effectively utilised as a management tool that will be beneficial for minimising such losses of raw material quality post-harvest.

The DSS system will draw information from different scientific areas including the need of better knowledge of the mycobiome at harvest in both dry and wet years. Studies at Cranfield have previously identified that because cereals are respiring, changes in CO₂ can be a very early indicator of poor hygiene due to initiation of mould growth and increased risks of mycotoxin contamination, especially related to zearalenone, ochratoxin A, and T-2 and HT-2 toxin (in oats) (Garcia-Cela et al., 2018). Studies are needed to evaluate the initial mycobiome, and kinetic changes during drying and storage. Our data has also previously shown that dry matter losses of <1.0% can result in an exceedance of the prevailing legislative limits for these and other toxins (Garcia-Cela et al., 2019). The student developing this project will work with the hypothesis that by identifying and quantifying both the mycobiome and the mycotoxin profile (as part of the fungal metabolome) at harvest, using a combination of CO₂ measurements in store and linking this to biological models on boundary conditions for growth and mycotoxin production, it will be possible to develop an integrated post-harvest DSS for improved management of stored cereals and reduce waste streams.

Lead Supervisor

Appendices

Title	First Name	Surname
<input type="text" value="Dr"/>	Angel	Medina
School	<input type="text" value="School of Water, Energy and Environment"/>	
Email	<input type="text" value="a.medinavaya@cranfield.ac.uk"/>	

If the supervisor details above are missing or incorrect, please contact cures-support@cranfield.ac.uk and we will add or amend the information.

Do not sign the form in Part 6 until we have replied to you that the supervisor information has been updated.

Intended start date of data collection

Please note: you must not begin your research until approval has been given by CURES.

Will the research be sponsored or funded by an external organisation?

Y e s

Please enter the name of the external organisation

Please click on 'Next' in left hand actions bar to move forward to Part 2

Part 2: Ethical Risk

The following questions will help determine the level of ethical risk that your project entails.

Appendices

Please select any that apply:

Living animals are involved in my research

The research will use tissues from deceased animals that have been killed as part of the research project

The research involves living organisms/biological agents and could potentially impact other entities outside the laboratory

Participants may be seriously harmed in the course of the research

The proposed research could adversely affect the reputation of the university

The research data is about illegal activities or will be collected from those engaged in illegal activities, or has itself been collected illegally

None of the above

Please select any that apply:

The research will involve the collection and use of 'relevant material' under the Human Tissue Act 2004

There are significant power differences present, or dual or other complicating relationships exist

The research will deal with material that is obscene or violent in content

The risk is greater to the participant(s) than they would normally experience in their daily life (physical, emotional, psychological, career, financial)

The research interacts with members of potentially vulnerable groups (e.g. children, the elderly, those with learning difficulties, prisoners etc.)

National infrastructure will be impacted negatively (e.g. power grid)

None of the above

Please select any that apply:

The research involves interviews with or surveys of human participants where sensitive personal data will be gathered

The research involves interviews with or surveys of human participants where commercially sensitive data will be gathered

The research will use tissues from deceased animals that have come from the food supply chain, roadkill or an authorised source

The research will use tissues from living animals that have come from normal husbandry, a therapeutic routine, or operation

The research has defence or security implications

The research is based on unpublished material

None of the above

Please select any that apply:

Appendices If your research involves surveys or interviews with human participants who are fully able to provide their informed consent, and if applicable, organisational approval has been obtained for the employees of the organisation concerned to take part in the research

The research consists of interviews, where any published or recorded material does not contain personal data, unless permission has been obtained for its use (for example, name, job title or years in post)

Sensitive personal data will not be collected

The research consists of surveys, which will not collect personal data or identifying information

None of the above

If you are using animal tissue, please explain where it has come from. If it originates from an animal house provide the details of the university or other establishment in the box underneath and how they comply with the required Home Office approval; and under what license conditions

With reference to your answers above, please confirm whether your research requires obtaining informed consent

Yes

No

not applicable

Please explain why consent is not required for your research

Research does not involve participant engagement

Will participants be able, during the data gathering phase, to freely withdraw or modify their consent and to ask for the destruction of all or part of the data that they have contributed?

Yes

No

not applicable

Will your research adhere to the duty of confidentiality?

Yes

No

Not applicable

Are there any conflicts of interest?

Appendices

Yes

No

Not applicable

Are you collecting commercially sensitive data?

Yes

...

Please comment on any other ethical issues that may arise from your project

Please click on 'Next' in left hand actions bar to move forward to Part 3

Part 3: Methodology and Expertise

By completing the following information on your research methodology will help us match your application with the most appropriate reviewer(s).

What type of research design will you be using? (tick all that apply)

Experimental

Quasi-experimental

Cross-sectional or survey

Longitudinal

Case study

Comparative

What type of research strategy will you employ?

Qualitative

Quantitative

Mixed methods

Which methods will you be using? (tick all that apply)

Appendices

Experimental - design
Experimental - field
Experimental - laboratory
Randomized controlled trial / other intervention study
Interview
Observation (for human participants only)
Focus groups
Questionnaires/surveys
Action research
Personal documents
Medical records
Literature review
Systematic review
Secondary data analysis
Advisory/consultation/collaborative groups
Computational
Other

Please describe briefly the population that you will study

N/A

How will you evaluate (validate/verify/analyse) your data?

I will collect the data and then determine whether there are significant groupings or trends present (information-theoretic models)

I will test a hypothesis

Please assign your research project to one of the University Themes

Environment and Agrifood

Please click on 'Next' in left hand actions bar to move forward to Part 4

Part 4: Specialist Studies

This section relates to research around health and defence topics. For most applications the questions will not be applicable.

Have you ensured that your study complies with The Human Tissue Act 2004 and its nine codes of practice?

Appendices

Yes

No

Not applicable

Are you conducting research involving human participants undertaken, funded or sponsored by the Ministry of Defence?

Yes

No

Is the research taking place in or through the National Health Service (NHS)?

Yes

Please click on 'Next' in left hand actions bar to move forward to Part 5

Part 5: Supporting Documents

You are encouraged to upload a research protocol if this is common practice in your area of study. In addition, please upload any supporting documents you may have that will help reviewers understand your research design such as questionnaires, interview schedules, participant information sheets, consent forms, case for support etc. (dependent on study)

Please click on 'Next' in left hand actions bar to move forward to Part 6

Part 6: Declarations and Signatures

Researcher Declaration

The completed form is accurate to the best of my knowledge and belief.

I undertake to abide by Cranfield University's [Ethics Policy](#) in undertaking this project.

I confirm that I have completed the Cranfield University Ethics Training successfully.

I understand that I am responsible for obtaining ethical approval and any other necessary approvals for my project.

I understand that I must not begin the data collection until I have received approval from Cranfield University Research Ethics System (CURES).

I understand that if any significant changes are subsequently made to my project then I will have to submit a new ethics application and until such time as new ethics approval is granted I will have to stop data collection on my project.

Researcher Signature

Appendices

Signed: This form was signed by Mrs Abimbola Oluwakayode (Abimbola.Oluwakayode@cranfield.ac.uk) on 13/10/2020 13:10

Supervisor Declaration

I confirm that I have read and fully support this application and will be acting as the supervisor of the lead researcher (student) for this project.

I have checked that the application has been completed correctly and is of good quality.

In my opinion, the research proposal is viable.

I confirm that my student has been instructed to complete the Cranfield University Ethics Training.

I understand that the lead researcher I am supervising must not begin the data collection until they have received approval from the Cranfield University Research Ethics System (CURES).

I understand that if any significant changes are subsequently made to this project then the lead researcher will be required to submit a new ethics application and until such time as new ethics approval is granted they must stop data collection on the project.

I understand that if any of the above conditions are not met then the University reserves the right to refuse to assess the student's work for any intended degree

Lead Supervisor Signature

Signed: This form was signed by Professor Angel Medina Vaya (a.medinavaya@cranfield.ac.uk) on 20/01/2021 10:56

Students must obtain a signature from their supervisor.

Once your supervisor has signed the form, your application will be automatically submitted.

Appendix B Supplementary data for Chapter 2

B.1.1 Moisture and Water Activity of Grains

Table B-1: Average values ($n=3 \pm \text{SE}$) of moisture content and water activity of grains.

Samples	Moisture Content (%)	SE	Water Activity	SE
WHEAT ENG '19	11.69	0.00	0.65	0.00
WHEAT ENG '20	13.27	0.01	0.67	0.01
WHEAT ENG '21	16.07	0.00	0.79	0.00
WHEAT NI '20 1	12.31	0.04	0.6	0.00
WHEAT NI '20 2	13.42	0.03	0.72	0.00
BARLEY ENG '19	14.78	0.01	0.77	0.00
BARLEY ENG '20 1	14.01	0.06	0.73	0.01
BARLEY ENG '20 2	10.98	0.16	0.57	0.01
BARLEY ENG '21 1	10.15	0.02	0.58	0.00
BARLEY ENG '21 2	12.88	0.01	0.75	0.00
BARLEY NI '20	13.18	0.09	0.63	0.02
MAIZE ENG '19	14.53	0.09	0.79	0.01
MAIZE NI '20	11.69	0.10	0.5	0.01
OAT ENG '19	13.58	0.01	0.72	0.00
OAT ENG '21 1	13.58	0.05	0.74	0.00
OAT ENG '21 2	12.23	0.05	0.73	0.00
OAT NI '21 1	5.10	0.01	0.26	0.03
OAT NI '21 2	4.80	0.00	0.22	0.02
OAT NI '21 3	5.93	0.02	0.26	0.00
OAT NI '21 4	6.69	0.02	0.29	0.00

SE: Standard error

B.2 Fungal populations in grains

Table B-2: Mean values ($n=3 \pm \text{SD}$) of the initial fungal populations ($\log_{10}$ CFUs/g) in the grains.

Grains	<i>Penicillium</i>		<i>Fusarium</i>		<i>A. section Nigri</i>	
	MEA+	DG18+	MEA+	DG18+	MEA+	DG18+
WHEAT ENG '19	3.7 ± 1.8	3.6 ± 1.9	0.0 ± 0.0	2.9 ± 1.7	0.0 ± 0.0	0.0 ± 0.0
WHEAT ENG '20	3.3 ± 1.9	3.4 ± 1.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
WHEAT ENG '21	2.6 ± 1.6	2.6 ± 1.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
WHEAT NI '20	2.8 ± 1.7	2.8 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
BARLEY ENG '19	2.9 ± 0.0	3.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
BARLEY ENG '20	2.6 ± 0.7	2.3 ± 2.2	3.6 ± 3.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
BARLEY ENG '21	5.6 ± 5.6	5.6 ± 5.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
BARLEY NI '20	3.1 ± 0.0	3.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.1 ± 0.0	0.0 ± 0.0
OAT ENG '19	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
OAT ENG '21	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
OAT NI '21	5.3 ± 5.2	5.5 ± 5.5	0.0 ± 0.0	2.3 ± 2.0	0.0 ± 0.0	0.0 ± 0.0
MAIZE ENG '19	2.6 ± 2.5	2.6 ± 2.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MAIZE NI '20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

SD: Standard deviation. MEA+: Malt Extract Agar media. DG18+: Dichloran Glycerol Agar media. +: chloramphenicol. ENG: England, NI- Northern Ireland. '19, '20 and '21- years 2019, 2020 and 2021.

Appendices

Table B-3: Mean values ($n = 5 \pm \text{SD}$) of the initial fungal isolations in the grains

Grains	<i>Penicillium</i>				<i>Fusarium</i>				<i>A. section Nigri</i>			
	MEA+		DG18+		MEA+		DG18+		MEA+		DG18+	
	NSS	SS	NSS	SS	NSS	SS	NSS	SS	NSS	SS	NSS	SS
WHEAT ENG '19	80	4	100	16	0	0	0	88	4	0	4	0
WHEAT ENG '20	80	8	100	20	4	0	0	68	8	0	0	0
WHEAT ENG '21	0	4	48	0	0	0	76	96	0	0	0	0
WHEAT NI '20	52	6	84	4	4	2	8	58	2	0	12	0
BARLEY ENG '19	8	0	88	24	0	0	0	0	0	0	0	0
BARLEY ENG '20	16	0	86	14	54	22	12	0	0	0	0	0
BARLEY ENG '21	4	8	84	24	0	0	0	0	4	0	16	0
BARLEY NI '20	54	6	90	20	0	0	68	92	0	0	0	0
OAT ENG '19	16	0	48	0	76	4	28	36	4	0	0	0
OAT ENG '21	12	0	84	0	0	0	40	68	0	0	0	0
OAT NI '21	44	8	61	15	2	0	38	76	0	0	1	0
MAIZE ENG '19	0	36	88	72	0	0	0	0	0	12	12	0
MAIZE NI '20	32	0	100	0	0	0	0	0	0	0	0	0

SD: Standard deviation. MEA+: Malt Extract Agar media. DG18+: Dichloran Glycerol Agar media. +: chloramphenicol. SS-surface sterilisation. NSS- non-surface sterilisation. ENG: England, NI- Northern Ireland. '19, '20 and '21- years 2019, 2020 and 2021.

MAIZE NI '20- has 4% of *A. section Flavi* on MEA+ and NSS

BARLEY ENG '20 has 2% of *A. section Flavi* on DG18+ and NSS

Appendix C Supplementary data for Chapter 3

C.1 Visual representation of irradiated oats

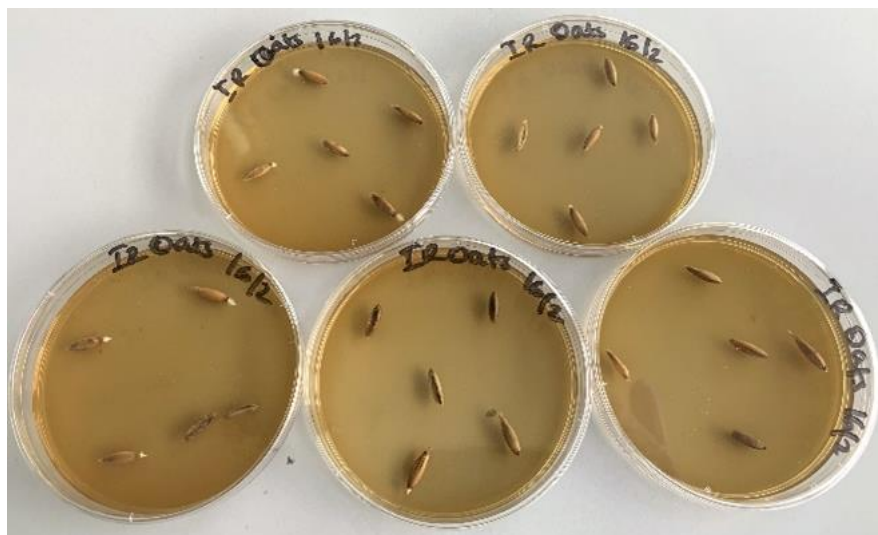


Figure C-1: Pictorial representations of irradiated oat grains on Malt Extract Agar (MEA+) +: chloramphenicol after seven days of incubation.

Table C-1: The measurement uncertainty for each analyte

Analytes	2SD	3SD	Mean Conc. (µg/kg)	Ur (%)	U (%)
Moniliformin	22.2	33.3	174	13	19
15-Acetyldeoxynivalenol	30.2	45.2	114	26	40
3-Acetyldeoxynivalenol	10.1	15.1	190	5	8
Beauvericin	10.2	15.3	93.8	11	16
Diacetoxyscirpenol	20.0	30.0	187	11	16
Deoxynivalenol	12.3	18.5	191	6	10
DON-3-G	6.5	9.7	69.0	9	14
Enniatin A	11.5	17.3	85.9	13	20
Enniatin A1	9.5	14.3	88.8	11	16
Enniatin B	16.8	25.2	97.5	17	26
Enniatin B1	13.4	20.1	93.8	14	21

Appendices

HT-2 toxin	9.6	14.5	188	5	8
HT-2-Glc	9.9	14.9	192	5	8
Nivalenol	71.7	108	160	45	67
T-2 toxin	9.0	13.4	187	5	7

SD- Standard Deviation. Conc.- Concentration. Ur: Relative expanded measurement uncertainty estimated from intra-laboratory validation (intermediate precision) data based on five lots of oats, close to a 95% confidence interval. U: Relative expanded measurement uncertainty estimated from intra-laboratory validation (intermediate precision) data based on five lots of oats, close to a 99% confidence interval.

C.1.1 Temperature x a_w impacts on mycotoxins

Table C-2: Statistical differences in the concentrations of each trichothecene at all storage conditions using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.

Treatments		Levels	T-2 toxin	HT-2 toxin	HT-2-Glc	DON	DON-3-G	DAS
Naturally contaminated oat control	aw	One-way ANOVA	0.3660	0.2225	0.6449	0.0009	0.0015	0.0198
		0.98-0.95	0.6721	0.3184	0.9575	0.0009	0.0009	0.0135
	T [°C]	One-way ANOVA	0.4929	0.2784	0.3600	0.2483	0.2758	0.2031
		25- 20	0.2237	1.0000	0.7899	0.3717	0.3184	0.4005
Naturally contaminated oat + <i>F. langsethiae</i>	aw	One-way ANOVA	0.2930	0.3516	0.3834	<.0001	0.0001	0.5608
		0.98-0.95	0.1036	0.5635	0.3706	0.0009	0.0009	0.3184
	T [°C]	One-way ANOVA	0.547	0.2673	0.2120	0.4350	0.2386	0.4931
		25- 20	0.0661	0.7132	0.6355	0.4948	0.4309	0.6365
Irradiated oat control	aw	One-way ANOVA	0.0015	<.0001	0.0072	0.0003	<.0001	0.1489
		0.98-0.95	0.0122	0.0008	0.0116	0.0004	0.0004	0.1709

Appendices

	T [°C]	One-way ANOVA	0.1362	0.5863	0.0383	0.5082	0.9093	0.9914
		25- 20	0.2202	0.7091	0.1071	0.7791	0.9553	1.0000
Irradiated oat + <i>F. langsethiae</i>	aw	One-way ANOVA	0.0662	<.0001	<.0001	0.2383	0.0422	0.9257
		0.98-0.95	0.0831	0.0009	0.0009	0.2271	0.0831	0.4309
	T [°C]	One-way ANOVA	0.0307	0.9702	0.3185	0.1389	0.6538	0.0337
		25- 20	0.0313	0.6365	0.2271	0.0074	0.9581	0.1036

Table C-3: Statistical differences in the concentrations of trichothecenes at each water activity and temperature for all treatments using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method

		20 °C				25°C			
Treatments	aw Levels	One-way ANOVA	T-2: HT2- Glc	HT-2: HT2- Glc	T-2: HT-2	One-way ANOVA	T-2: HT2- Glc	HT-2: HT2- Glc	T-2: HT-2
Naturally contaminated oat control	0.95	0.5718	1.000	0.4678	0.3123	0.2139	0.3123	0.8852	0.0304
	0.98	0.1418	0.1241	0.3836	0.1241	0.4106	0.4705	0.6650	0.3123
Naturally contaminated oat + <i>F. langsethiae</i>	0.95	0.4139	1.0000	0.4705	0.4705	0.1538	0.0304	0.6650	0.0304
	0.98	0.6135	1.0000	1.0000	0.6650	0.1682	0.0304	0.3123	0.1939
Irradiated oat control	0.95	0.0131	0.0131	0.0131	0.0131	0.0131	0.0131	0.0131	0.0131
	0.98	0.5275	0.1832	0.1832	1.0000	0.4426	0.8852	0.3123	0.4705
Irradiated oat + <i>F. langsethiae</i>	0.95	0.0009	0.0304	0.0304	0.0304	<.0001	0.0304	0.0304	0.0304
	0.98	0.7799	0.4705	1.0000	0.4705	0.0071	0.6650	0.0304	0.0304

Appendices

Table C-4: Influence of a_w x temperature on the concentrations of Diacetoxyscirpenol in oats.

Mycotoxins (ng/g of grains) ^a			
Treatments	a_w	20 °C	25 °C
Naturally contaminated oat control	0.95	12	8
	0.98	7	< LOD
Naturally contaminated oat + <i>F. langsethiae</i>	0.95	7	< LOQ
	0.98	< LOQ	< LOQ
Irradiated oat control	0.95	< LOQ	< LOQ
	0.98	< LOD	< LOD
Irradiated oat + <i>F. langsethiae</i>	0.95	19*	9*
	0.98	19*	9*

Table C-5: Statistical differences in the concentrations of DON-3-G to DON at each water activity and temperature for all treatments using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.

Mycotoxins	NOC			NOFL		IOC		IOFL	
	a_w , T [°C]	ANOVA	Wilcoxon	ANOVA	Wilcoxon	ANOVA	Wilcoxon	ANOVA	Wilcoxon
DON-3-G: DON	0.95, 20	0.3049	0.1939	0.0013	0.0304	1.0000	1.0000	0.0008	0.0304
DON-3-G: DON	0.95, 25	0.3709	1.0000	0.0034	0.0304	1.0000	1.0000	0.0024	0.0304
DON-3-G: DON	0.98, 20	0.0760	0.1939	0.0054	0.0304	0.0079	0.0304	0.1241	0.0304
DON-3-G: DON	0.98, 25	0.0031	0.0304	0.0124	0.0304	0.0536	0.0304	0.0080	0.0304

Appendix D Supplementary data for Chapter 4

D.1 The method validation.

Table D-1: Validation spiking levels in the wheat

Mycotoxin	Level 1 (ng/g)	Level 2 (ng/g)	Level 3 (ng/g)
15-Acetyl-DON	50	100	150
3-Acetyl-DON	50	100	150
Enniatin A	50	100	150
Enniatin A1	50	100	150
Enniatin B	50	100	150
Enniatin B1	50	100	150
Nivalenol	50	100	150
ZEN-14-Glucoside (ZEN-14-G)	50	100	150
ZEN-16-Glucoside (ZEN-16-G)	50	100	150
ZEN-14-Sulphate (ZEN-14-S)	50	100	150
Moniliformin (MON)	50	100	150
DON-3-G	50	100	150
Zearalenone (ZEN)	100	200	300
Deoxynivalenol (DON)	100	200	300
Diacetoxyscirpenaol (DAS)	25	50	75

Note: All at 0.5, 1 and 1.5 times a particular level (Level 2) to replicate the EC directive

Appendices

Table D-2: The measurement uncertainty for each analyte

Analyte	SD	2.SD	3.SD	Conc. (ng/g)	U _r (%)	U (%)
MON	1.7	3.3	5.0	35.1	9%	14%
15-AcDON	1.8	3.7	5.5	41.2	9%	13%
3-AcDON	1.4	2.8	4.2	34.0	8%	12%
DAS	1.3	2.6	3.9	25.7	10%	15%
DON	3.9	7.8	11.7	103.5	8%	11%
DON-3-G	2.0	3.9	5.9	47.4	8%	12%
Enniatin A	1.4	2.8	4.2	42.4	7%	10%
Enniatin A1	2.1	4.3	6.4	49.2	9%	13%
Enniatin B	3.6	7.2	10.7	61.1	12%	18%
Enniatin B1	2.3	4.6	6.9	54.9	8%	13%
Nivalenol	3.5	6.9	10.4	53.4	13%	19%
Zearalenone	2.7	5.3	8.0	100.1	5%	8%
ZEN-14-G	1.7	3.4	5.0	53.8	6%	9%
ZEN-14-S	1.4	2.8	4.2	17.7	16%	24%
ZEN-16-G	2.2	4.3	6.5	55.6	8%	12%

SD- Standard Deviation.

U_r: Relative expanded measurement uncertainty estimated from intra-laboratory validation (intermediate precision) data based on nine replicates of wheat, close to a 95% confidence interval.

U: Relative expanded measurement uncertainty estimated from intra-laboratory validation (intermediate precision) data based on nine replicates of wheat, close to a 99% confidence interval.

Appendices

Table D-3: Mean values (n=9) of the Inter-Day Precision within lab reproducibility (WLR) method validation for wheat grains.

Component Name	Level 1				Level 2				Level 3			
	Conc. (ng/g)	SD	RSDWLR	RAWLR (%)	Conc. (ng/g)	SD	RSDWLR	RAWLR (%)	Conc. (ng/g)	SD	RSDWLR	RAWLR (%)
MON	35.1	1.7	4.7%	70.2%	72.3	1.9	2.7%	72.3%	111.7	3.1	2.7%	74.4%
15-AcDON	41.2	1.8	4.4%	82.5%	74.1	2.3	3.1%	74.1%	111.6	5.0	4.5%	74.4%
3-AcDON	34.0	1.4	4.1%	68.1%	68.8	3.3	4.8%	68.8%	103.9	4.9	4.7%	69.2%
DAS	25.7	1.3	5.1%	102.8%	50.5	1.8	3.5%	101.1%	75.0	1.9	2.5%	100.0%
DON	103.5	3.9	3.8%	103.5%	196.5	6.8	3.5%	98.3%	283.0	10.4	3.7%	94.3%
DON-3-G q	47.4	2.0	4.1%	94.7%	90.9	4.4	4.8%	90.9%	137.1	3.4	2.5%	91.4%
Enniatin A	42.4	1.4	3.3%	84.8%	87.5	2.2	2.6%	87.5%	132.1	2.3	1.7%	88.1%
Enniatin A1	49.2	2.1	4.4%	98.3%	97.4	3.6	3.7%	97.4%	145.8	4.2	2.9%	97.2%
Enniatin B	61.1	3.6	5.9%	122.2%	95.4	5.0	5.2%	95.4%	141.7	4.4	3.1%	94.5%
Enniatin B1	54.9	2.3	4.2%	109.7%	96.0	4.2	4.3%	96.0%	144.6	3.2	2.2%	96.4%
Nivalenol	53.4	3.5	6.5%	106.9%	96.9	3.6	3.7%	96.9%	139.4	8.2	5.9%	92.9%
Zearalenone	100.1	2.7	2.7%	100.1%	190.6	6.7	3.5%	95.3%	282.5	10.2	3.6%	94.2%
ZEN-14-G	53.8	1.7	3.1%	107.5%	107.1	4.1	3.8%	107.1%	161.1	5.4	3.4%	107.4%
ZEN-14-S	17.7	1.4	8.0%	35.4%	33.3	1.8	5.4%	33.3%	51.1	3.0	5.9%	34.1%
ZEN-16-G	55.6	2.2	3.9%	111.2%	111.7	3.6	3.2%	111.7%	167.3	5.9	3.5%	111.5%

WLR- Within lab Reproducibility. RSD- Relative Standard Deviation. RA- Apparent Recovery. SD- Standard Deviation. Conc.- Concentration.

Appendices

Table D-4: Statistical differences in the concentrations of each analyte at all storage conditions using one-way ANOVA and nonparametric comparisons for each pair using Wilcoxon method.

		Levels	DON	DON-3-G	ZEN	ZEN-14-S	ZEN-16-G	ZEN-14-G		
Naturally contaminated wheat control	a _w	One-way ANOVA	0.0876	0.0012	0.2449	0.2083	0.1943	-		
		0.98- 0.95	0.0136	1.0000	0.0066	0.0037	0.7091	1.0000		
		0.98- 0.93	0.0136	0.0133	0.1267	0.0015	0.3184	1.0000		
		0.95-0.93	0.0406	0.0009	0.0355	0.1709	0.1250	1.0000		
	T	One-way ANOVA	0.5338	0.1850	0.3873	0.4740	0.0001	-		
Natural wheat + <i>F. graminearum</i>	a _w	25- 20	0.6650	0.2598	0.1521	0.3358	0.0004	1.0000		
		One-way ANOVA	<.0001	<.0001	<.0001	0.0020	0.3659	0.0031		
		0.98- 0.95	0.0009	0.0009	0.0009	0.0009	0.9570	0.0159		
		0.98- 0.93	0.0009	0.0009	0.0009	0.0009	0.6946	0.0046		
	T	0.95-0.93	0.3720	0.8664	0.0101	0.0101	0.1773	0.1709		
		One-way ANOVA	0.8101	0.6321	0.4250	0.6474	0.0174	0.4945		
		25- 20	0.7508	0.7913	0.3408	0.9310	0.0194	0.4915		
		Irradiated wheat control	a _w	One-way ANOVA	0.0594	0.0314	0.0073	0.5263	0.0117	-
				0.98- 0.95	0.0100	0.0458	0.0018	0.0100	0.0004	1.0000
				0.98- 0.93	0.1033	0.0458	0.0004	0.0009	0.4275	1.0000
0.95-0.93	0.0323			0.6946	0.3816	0.2246	0.0375	1.0000		
T	One-way ANOVA		0.0622	0.0719	0.0414	0.1685	0.0069	-		
Irradiated wheat + <i>F. graminearum</i>	a _w	25- 20	0.0539	0.3297	0.3358	0.9274	0.0243	1.0000		
		One-way ANOVA	<.0001	<.0001	0.0005	0.1673	0.2226	<.0001		
		0.98- 0.95	0.0009	0.0009	0.0313	0.4002	0.4309	0.0135		
		0.98- 0.93	0.0009	0.0009	0.0009	1.0000	0.2271	0.0006		
	T	0.95-0.93	0.0009	0.2271	0.0039	1.0000	0.4948	0.0092		
		One-way ANOVA	0.2162	0.3590	0.0227	0.0891	0.1170	0.5381		
		25- 20	0.3123	0.6236	0.0262	<.0001	0.0999	0.2994		

Appendices

Table D-5: Statistical differences in the ratios of DON-3-G to DON at each water activity and temperature for all treatments using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.

Mycotoxins	A _w , T °C	NWC		NWFG		RWC		RWFG	
		ANOVA	Wilcoxon	ANOVA	Wilcoxon	ANOVA	Wilcoxon	ANOVA	Wilcoxon
DON-3-G: DON	0.93, 20	0.1207	0.1939	<0.0001	0.0304	0.2352	0.6446	0.0061	0.0304
DON-3-G: DON	0.93, 25	0.0069	0.0304	<0.0001	0.0211	0.0415	0.0294	0.2992	0.3123
DON-3-G: DON	0.95, 20	<0.0001	0.0304	0.0014	0.0211	0.0440	0.0689	0.0003	0.0304
DON-3-G: DON	0.95, 25	0.0013	0.0304	0.0143	0.0304	0.4015	1.0000	0.0041	0.0304
DON-3-G: DON	0.98, 20	0.2533	0.0384	0.0110	0.0304	0.1510	0.1939	0.0005	0.0304
DON-3-G: DON	0.98, 25	0.0711	0.0304	0.0039	0.0304	0.0651	0.0200	<0.0001	0.0304

NWC- Naturally contaminated wheat control. NWFG- Natural wheat + *F. graminearum*. RWC- Irradiated wheat control. RWFG- Irradiated wheat + *F. graminearum*

Table D-6: Statistical differences in the ratios of zearalenone (ZEN) to its conjugates at each water activity and temperature in the natural wheat control using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.

Mycotoxins	Naturally contaminated wheat control						
		0.93, 20	0.93, 25	0.95, 20	0.95, 25	0.98, 20	0.98, 25
	ANOVA	0.2232	0.0001	<.0001	0.0643	0.4948	0.05588
ZEN-16-G: ZEN-14-G	Wilcoxon	0.0211	0.0211	0.0211	1.0000	0.0689	0.0211
ZEN-16-G: ZEN-14-S	Wilcoxon	0.0211	0.0211	0.0211	0.1859	0.2454	0.0304
ZEN-16-G: ZEN	Wilcoxon	0.3094	0.0304	0.0211	0.0211	0.0304	0.3123
ZEN-14-S: ZEN-14-G	Wilcoxon	1.0000	1.0000	1.0000	0.1859	0.0689	0.0211
ZEN=14-G- ZEN	Wilcoxon	0.1859	0.0211	1.0000	0.0211	0.0211	0.0689
ZEN-14-S: ZEN	Wilcoxon	0.1859	0.0211	1.0000	0.8845	1.0000	0.1939

Appendices

Table D-7: Statistical differences in the ratios of zearalenone (ZEN) to its conjugates at each water activity and temperature in the natural wheat + *F. graminearum* using one-way ANOVA and nonparametric comparisons for each pair using Wilcoxon method.

Mycotoxins	Natural wheat + <i>F. graminearum</i>						
		0.93, 20	0.93, 25	0.95, 20	0.95, 25	0.98, 20	0.98, 25
	ANOVA	<.0001	0.2604	0.1291	0.0805	0.0145	0.0027
ZEN-16-G: ZEN-14-G	Wilcoxon	0.0211	1.0000	0.0211	0.6446	0.7715	0.1241
ZEN-16-G: ZEN-14-S	Wilcoxon	0.0304	0.0211	0.0304	0.0294	0.0304	0.0265
ZEN-16-G: ZEN	Wilcoxon	0.0304	0.0211	0.0304	0.0294	0.0304	0.0265
ZEN-14-S: ZEN-14-G	Wilcoxon	0.0211	0.0211	0.0211	0.0294	0.0304	0.0304
ZEN-14-G: ZEN	Wilcoxon	0.0211	0.0211	0.0211	0.0294	0.0304	0.0304
ZEN-14-S: ZEN	Wilcoxon	0.0304	0.6650	0.6650	0.8852	1.0000	0.1124

Table D-8: Statistical differences in the ratios of zearalenone (ZEN) to its conjugates at each water activity and temperature in the irradiated wheat control using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.

Mycotoxins	Irradiated wheat control						
		0.93, 20	0.93, 25	0.95, 20	0.95, 25	0.98, 20	0.98, 25
	ANOVA	<.0001	-	-	0.4299	0.0001	<.0001
ZEN-16-G: ZEN-14-G	Wilcoxon	0.0211	1.0000	1.0000	1.0000	0.0211	0.0211
ZEN-16-G: ZEN-14-S	Wilcoxon	0.0294	1.0000	1.0000	0.4533	0.6650	0.0606
ZEN-16-G: ZEN	Wilcoxon	0.0211	1.0000	1.0000	0.4533	0.0606	0.0211
ZEN-14-S: ZEN-14-G	Wilcoxon	0.0202	1.0000	1.0000	0.4533	0.0211	0.0211
ZEN-14-G: ZEN	Wilcoxon	1.0000	1.0000	1.0000	0.4533	0.0211	0.0304
ZEN-14-S: ZEN	Wilcoxon	0.0202	1.0000	1.0000	1.0000	0.0304	0.0304

Appendices

Table D-9: Statistical differences in the ratios of zearalenone (ZEN) to its conjugates at each water activity and temperature in the irradiated wheat + *F. graminearum* using one-way ANOVA and nonparametric comparisons for each pair using the Wilcoxon method.

Mycotoxins		Irradiated wheat+ <i>F. graminearum</i>					
		0.93, 20	0.93, 25	0.95, 20	0.95, 25	0.98, 20	0.98, 25
	ANOVA	0.008	<.0001	0.0352	0.0006	<.0001	<.0001
ZEN-16-G: ZEN-14-S	Wilcoxon	0.0211	0.3005	0.6573	0.0304	0.0304	0.0304
ZEN-16-G: ZEN	Wilcoxon	0.4705	0.0304	0.0689	0.0304	0.0211	0.0304
ZEN-16-G: ZEN	Wilcoxon	0.0304	0.0304	0.0304	0.0304	0.0304	0.0304
ZEN-14-S: ZEN-14-G	Wilcoxon	0.0211	0.0265	0.1859	0.0304	0.0211	0.0304
ZEN-14-G: ZEN	Wilcoxon	0.0211	0.0265	0.0294	0.0304	0.0304	0.0304
ZEN-14-S: ZEN	Wilcoxon	0.0304	0.0304	0.0211	0.0304	0.0211	0.0304

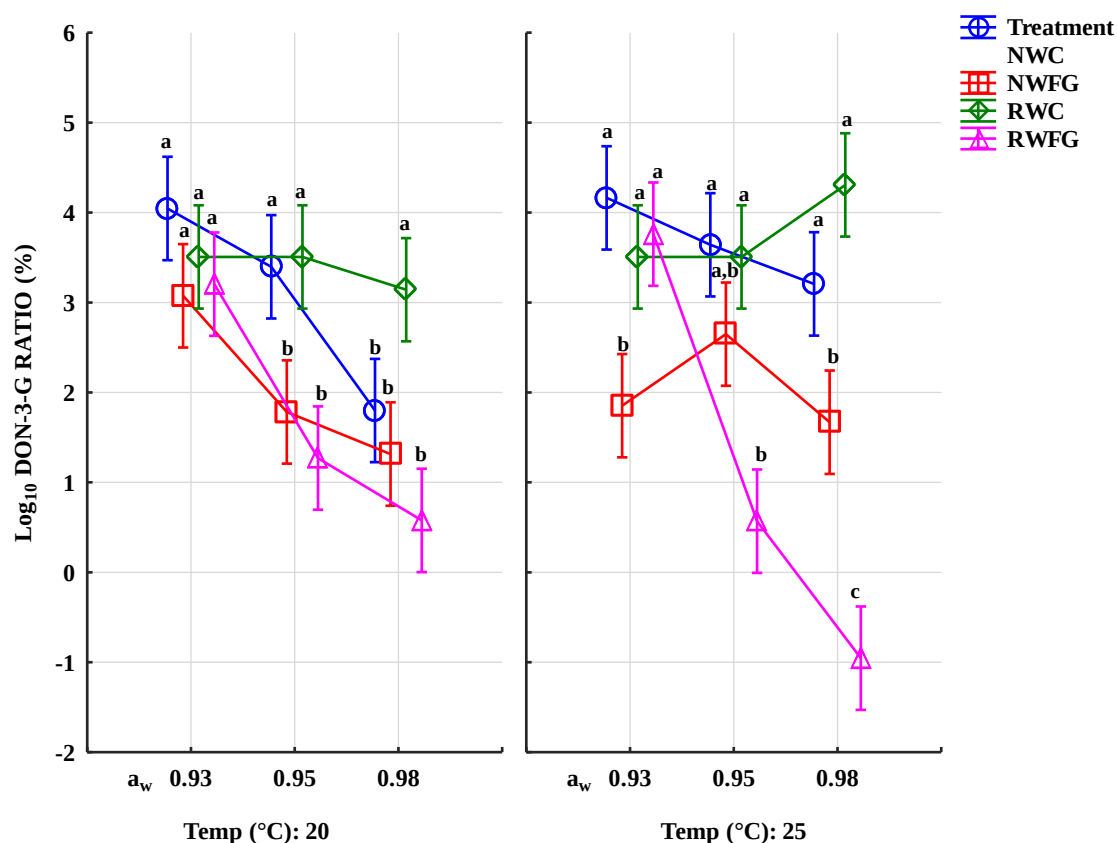


Figure D-1: DON-3-Glc concentration ratios (%) in all wheat treatments and all storage conditions. Vertical bars denote 0.95 confidence intervals. Different letters show significant differences in DON-3-Glc concentration ratios among all wheat treatments at each water activity level for each temperature using the Tukey HSD test. DON-3-Glc: Deoxynivalenol-3-glucoside. a_w: 0.93, 0.95 and 0.98. Temperature: 20 and 25 °C. NWC: naturally contaminated wheat control. NWFG: naturally contaminated wheat + *F. graminearum*. RWC: irradiated wheat control. RWFG: irradiated wheat + *F. graminearum*. Concentrations of analytes <LOD and <LOQ are assigned with values of LOD/2 and LOQ/2, respectively

DON-3-Glc concentration ratios were calculated using the Equation below.

$$[(\text{DON-3-Glc})/(\text{DON} + \text{DON-3-Glc})] \times 100$$

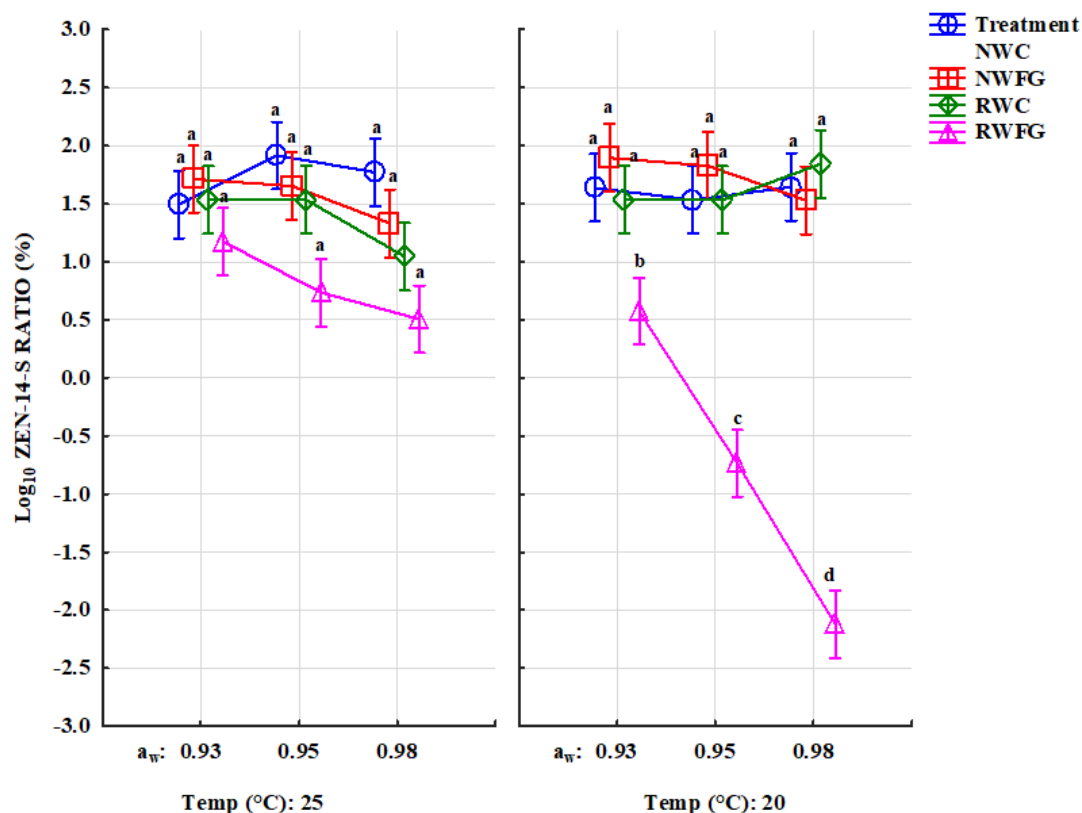


Figure D-2: ZEN-14-S concentration ratios (%) in all wheat treatments and all storage conditions. Vertical bars denote 0.95 confidence intervals. Different letters show significant differences in ZEN-14-S concentration ratios among all wheat treatments at each water activity level for each temperature using the Tukey HSD test. ZEN-14-S: Zearalenone-14-Sulphate. a_w : 0.93, 0.95 and 0.98. Temperature: 20 and 25 °C. NWC: naturally contaminated wheat control. NWFG: naturally contaminated wheat + *F. graminearum*. RWC: irradiated wheat control. RWFG: irradiated wheat + *F. graminearum*. Concentrations of analytes <LOD and <LOQ are assigned with values of LOD/2 and LOQ/2, respectively.

ZEN-14-S concentration ratios was calculated using the Equation below.

$$\left[\frac{\text{ZEN-14-S}}{\text{ZEN} + \text{ZEN-14-S}} \right] \times 100$$

Appendix E Supplementary data for Chapter 5

E.1 The respiration rates in naturally contaminated and *Penicillium verrucosum* inoculated wheat grains.

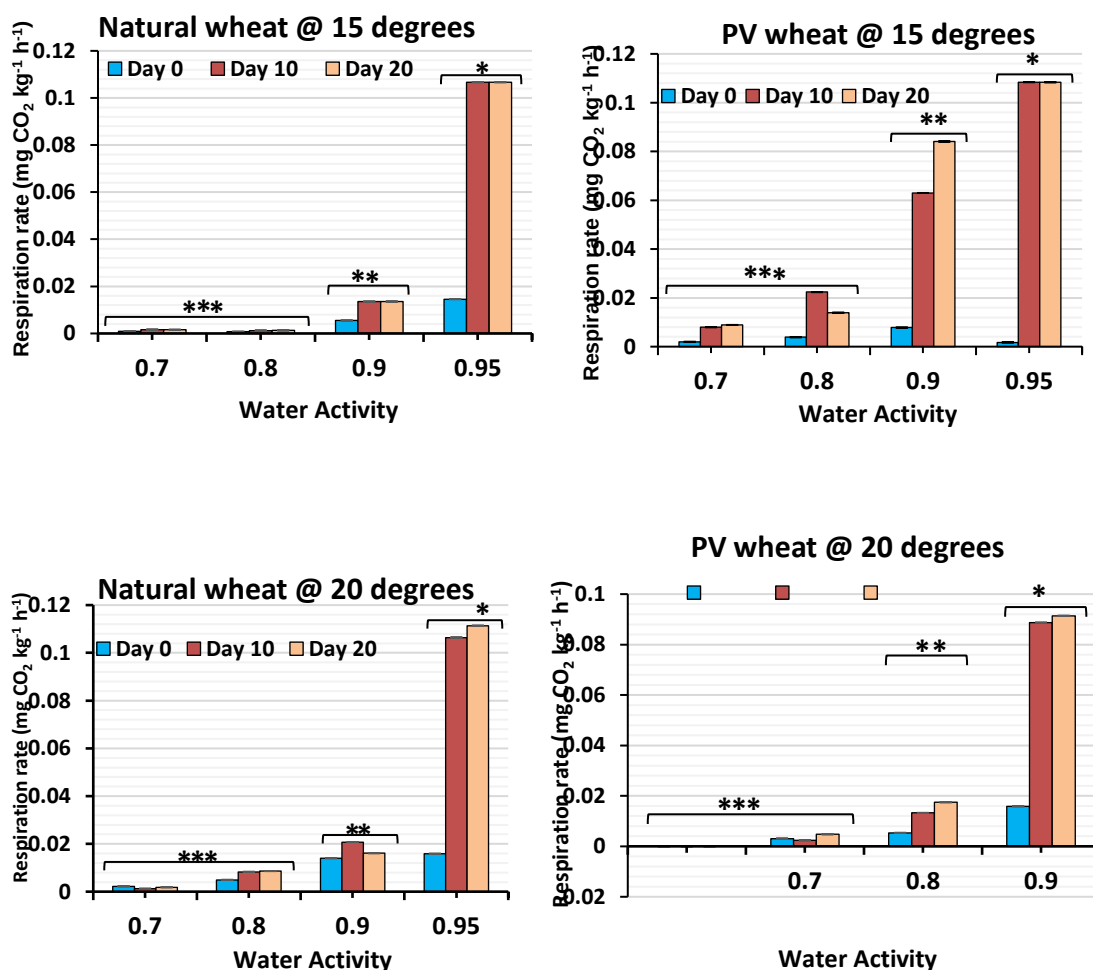


Figure E-1: Mean values of CO₂ respiration rates in naturally contaminated and *P. verrucosum* inoculated wheat at 15 and 20 °C for 20 days with standard error bars (n=2). Bars represent respiration rates at each storage day (day 0, day 10, and day 20) * shows significant differences between each water activity level in each treatment at each temperature using the Wilcoxon test (p <0.05). PV- *Penicillium verrucosum*.

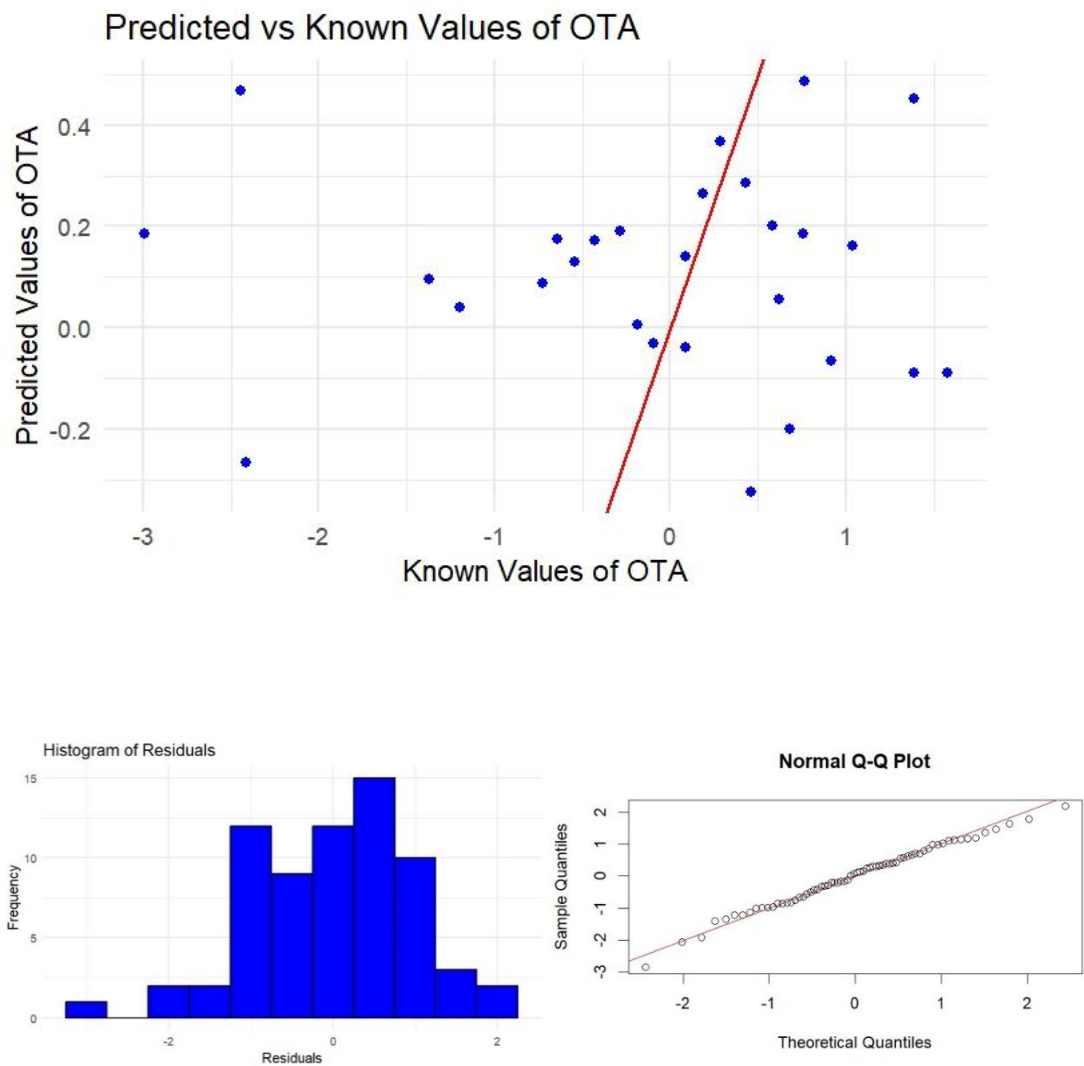


Figure E-2: Plot of the predicted against the known values of Ochratoxin A with residual plots

Table E-1: Heat map of correlations between CO₂ respiration rates, *Penicillium* populations, and ochratoxin concentrations in contaminated wheat for 0.90- 0.95 a_w .

Grains	Variables	OTA	OTB	OT α
Naturally contaminated wheat	CO ₂ RR	0.763504 (p= 0.000)	0.721640 (p= 0.000)	0.704578 (p= 0.000)
	<i>Penicillium</i> populations	-0.038969 (p= 0.832)	-0.007430 (p= 0.968)	-0.529732 (p= 0.002)
<i>Penicillium verrucosum</i> inoculated wheat	CO ₂ RR	0.101400 (p= 0.581)	0.103288 (p= 0.574)	0.341545 (p= 0.056)
	<i>Penicillium</i> populations	0.145544 (p= 0.427)	0.162596 (p= 0.374)	-0.357062 (p= 0.045)

Blue colour represents a positive correlation. Red colour represents a negative correlation. OTA- Ochratoxin A. OTB- Ochratoxin B. OT α - Ochratoxin alpha.CO₂ RR- Carbon dioxide respiration rates.

Appendix F Supplementary data for Chapter 6

F.1 Moisture adsorption curve for oat grains.

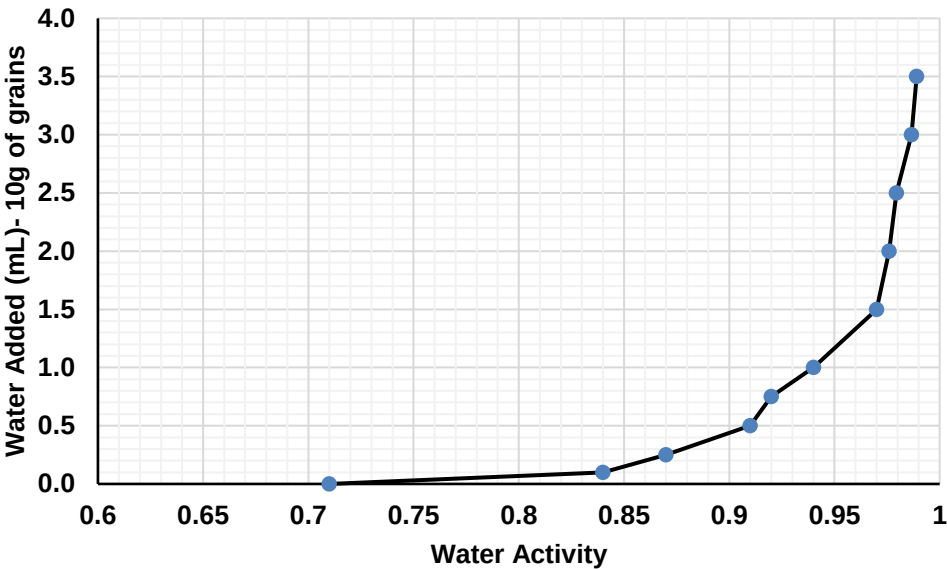
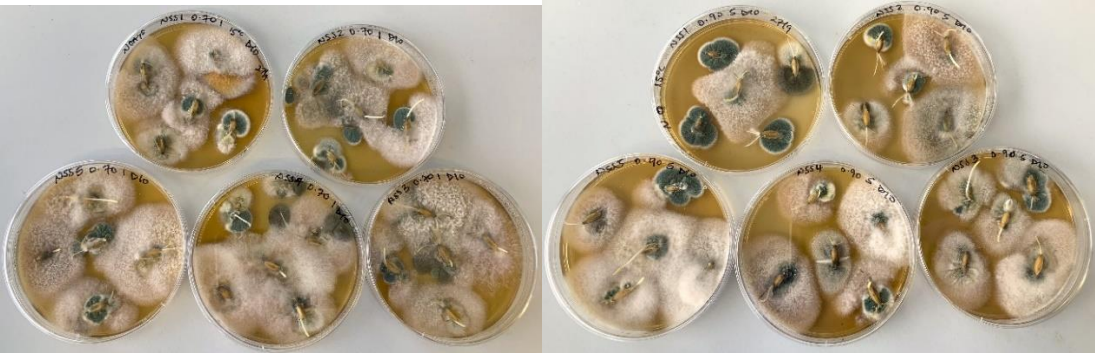


Figure F-1: Moisture Adsorption Curves for oat grains.

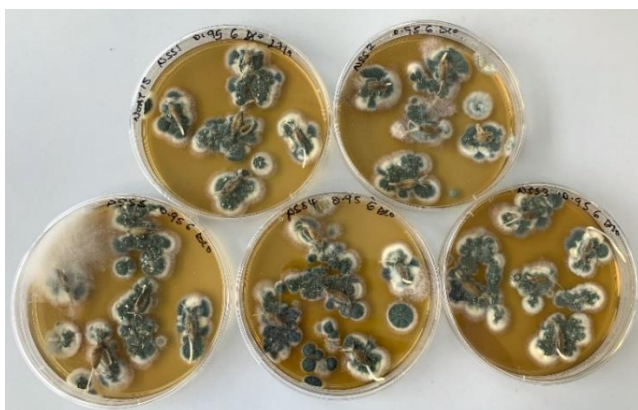
F.1.1 Visual representations of fungal contamination in stored oats.



NO, 0.70_{aw} and 15°C, DP

NO, 0.90_{aw} and 15°C, DP

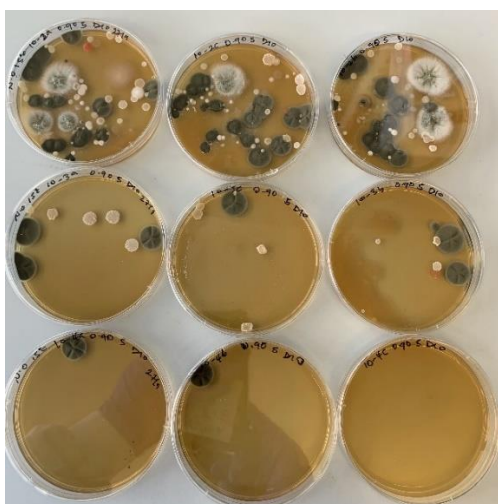
Appendices



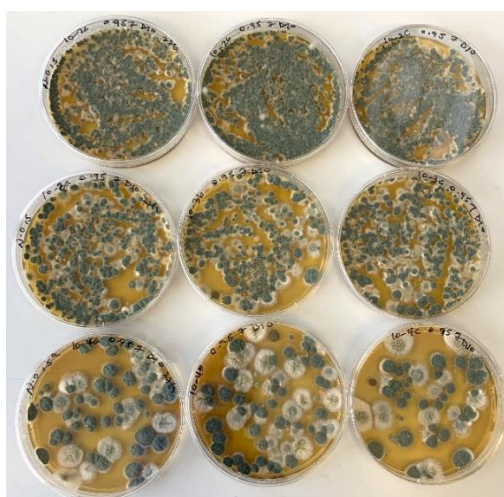
NO, 0.95 a_w and 15°C, DP



NO, 0.70 a_w and 15°C, SD



NO, 0.90 a_w and 15°C, SD



NO, 0.95 a_w and 15°C, SD

Figure F-2: Pictorial representation of fungal populations/isolations in contaminated oats on MEA+ media at all water activity (a_w) levels at 15°C for Direct plating (DP) and Serial dilution (SD) methods.

F.1.2 Respiration rates produced in stored oats at all storage conditions

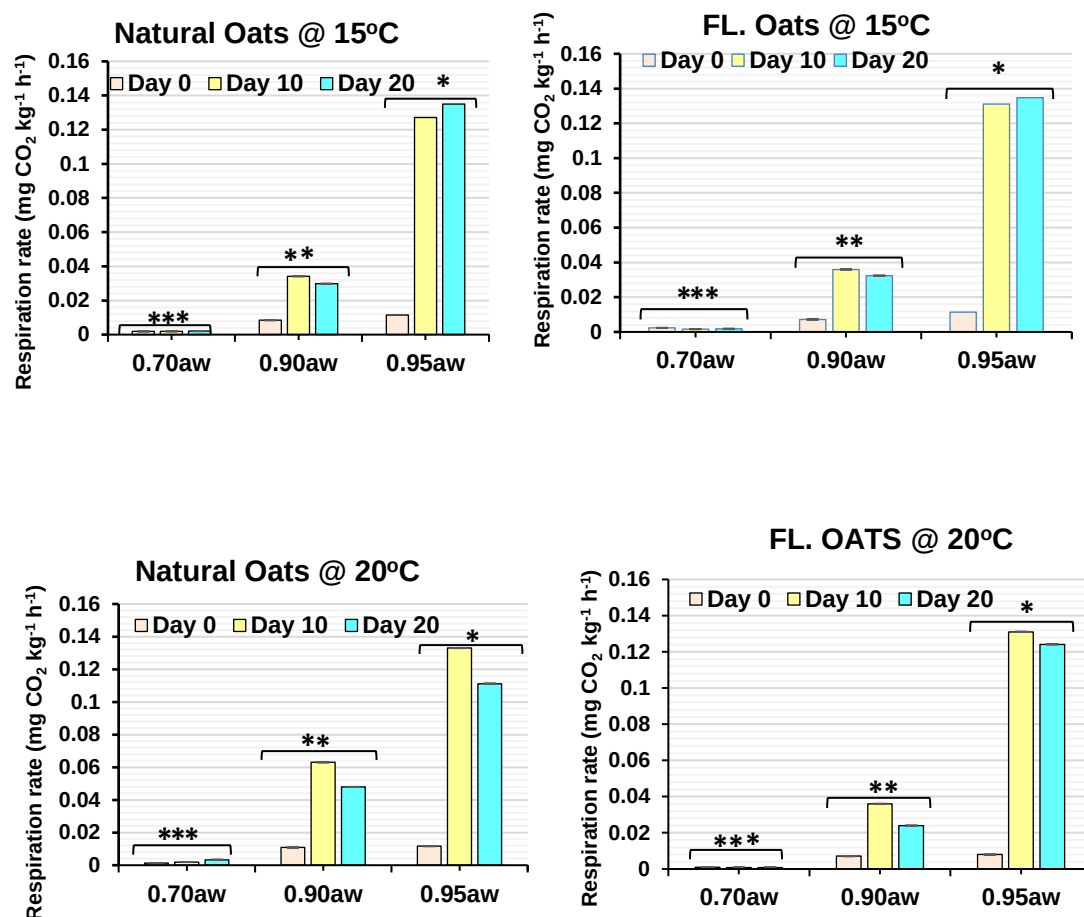


Figure F-3: Mean values of CO₂ respiration rates in contaminated oats at 15 and 20 °C for 20 days with standard error bars (n=3). Bars represent respiration rates at each storage day (day 0, day 10, and day 20) * shows significant differences between each water activity level at each temperature using the Wilcoxon test (p < 0.05). aw- water activity.

F.1.3 The Pearson Correlation coefficient (r) of respiration rates and mycotoxin concentrations in contaminated oats

Table F-1: Pearson Correlation coefficient (r) and p values of respiration rates and mycotoxin concentrations in contaminated oats.

Variable	Correlations. Marked correlations are significant at $p < .05000$ N=60						
	CO ₂	OTA	HT-2 Toxin	T-2 toxin	HT-2-Glc	Sum T2HT2HT2GLc	Sum t2ht2
CO ₂	1.0000	.5322	-.0916	-.1931	-.2902	.0039	-.0192
	p= ---	p=.000	p=.486	p=.139	p=.025	p=.976	p=.884
OTA	.5322	1.0000	-.0917	-.0763	-.2946	-.0009	.0483
	p=.000	p= ---	p=.486	p=.562	p=.022	p=.995	p=.714
HT-2 Toxin	-.0916	-.0917	1.0000	-.1646	.6274	.0181	.1194
	p=.486	p=.486	p= ---	p=.209	p=.000	p=.891	p=.364
T-2 toxin	-.1931	-.0763	-.1646	1.0000	-.1716	.8776	.8708
	p=.139	p=.562	p=.209	p= ---	p=.190	p=0.00	p=0.00
HT-2-Glc	-.2902	-.2946	.6274	-.1716	1.0000	-.0807	-.0963
	p=.025	p=.022	p=.000	p=.190	p= ---	p=.540	p=.464
Sum T2HT2HT2GLc	.0039	-.0009	.0181	.8776	-.0807	1.0000	.9799
	p=.976	p=.995	p=.891	p=0.00	p=.540	p= ---	p=0.00
Sum t2ht2	-.0192	.0483	.1194	.8708	-.0963	.9799	1.0000
	p=.884	p=.714	p=.364	p=0.00	p=.464	p=0.00	p= ---

F.1.4 Cross-validation and residual plots of the linear regression model for mycotoxins in oats

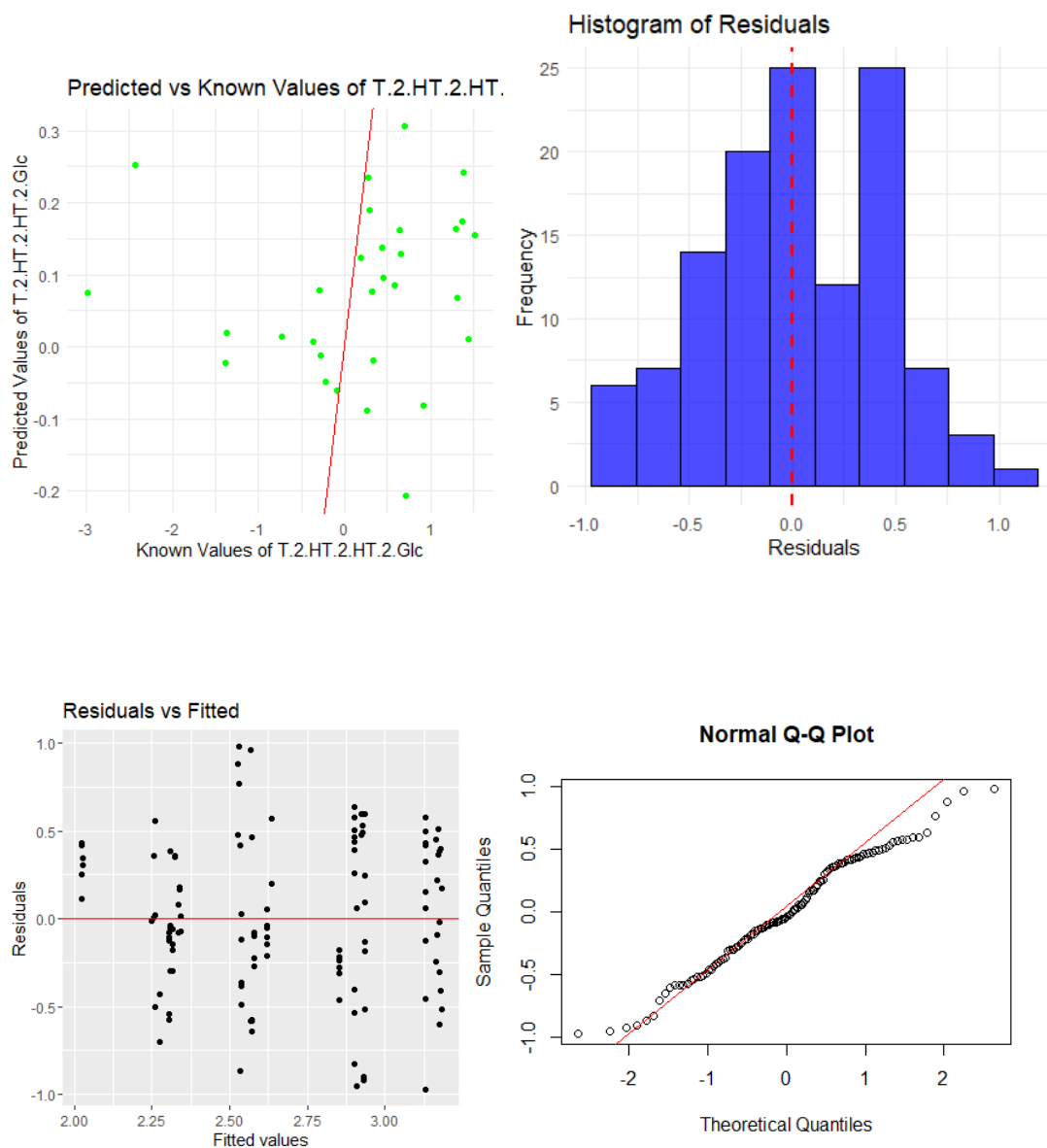


Figure F-4: Plot of the predicted against the known values of T-2+HT-2+HT-2-Glc with residuals and normal Q-Q plots.

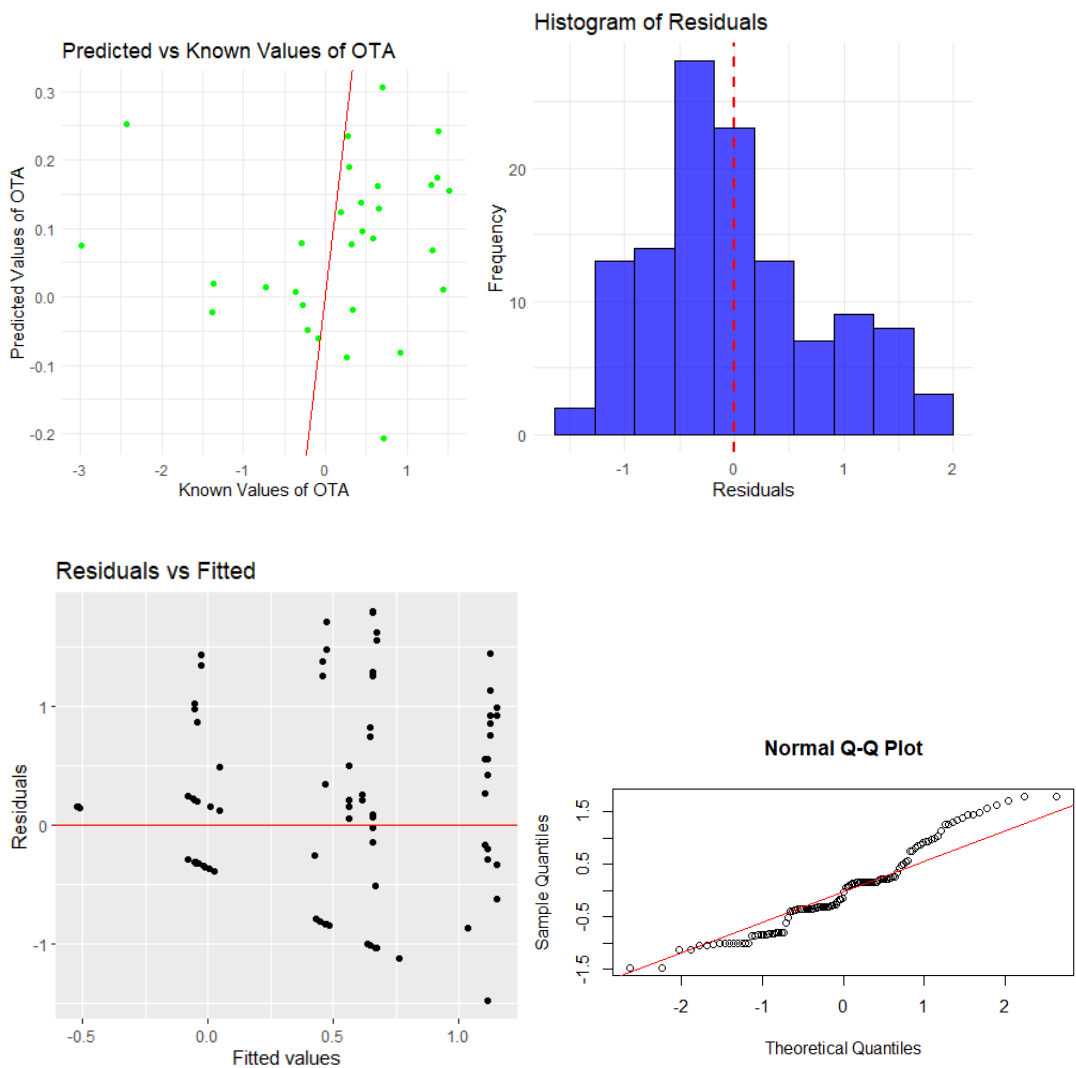


Figure F-5: Plot of the predicted against the known values of OTA with residuals and normal Q-Q plots