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Fate of Mycotoxins in Local-Race Populations of Maize Collected in South West of France, from the Field to the Flour and Meal in Organic Farms

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Abstract: Both organic and conventional farmers are confronted with the issue of mycotoxin contamination of maize, but organic farming is considered by the public to present a higher risk. There are also concerns about the sanitary quality of maize processed as a foodstuff and marketed on farms through short distribution channels, and there is a need of data on mycotoxin contaminations in such a farming system. With the objective to assess the diversity of contamination levels at harvest and to track the post-harvest fate of mycotoxins, maize grain samples were collected at organic farms from South West France after harvest, storage and milling. There was a wide range of levels of contamination by trichothecenes A and B, zearalenone, and fumonisins. The presence of ochratoxin A and aflatoxins was scarce. In some farms, but not all, the technique of drying and initial storage in cribs resulted in increased levels of contamination by *Fusarium* toxins, but not aflatoxins. The transfer of mycotoxins in milling products was higher for flour than for meal. Data are discussed in terms of mycotoxin co-occurrence, correlations between concentrations, and compliance with European Union regulations.

Keywords: Fusarium; corn; contaminant transfer

1. Introduction

Maize (*Zea mays* L.) is an important food security and income-generating crop in various world areas. Urge improvement in yields had been obtained by selection of hybrids, mechanisation, irrigation, use of mineral fertilizers and phytosanitary products, leading to an increase of the world production associated with global trades. Beside this agroeconomic system, farmers produce maize as a subsistence crop using local resources. Even in Europe, groups of organic farmers wishing to offer quality products, preserve cultivated biodiversity and the environment, and re-appropriate the age-old practice that is the self-production of seeds preserved and develop landrace populations. Landrace populations consist of a mixture of genotypes all of which are reasonably adapted to the region in which they evolved but which differ in detail such as reaction to water stress and soil fertility or to pests and diseases [1]. Among the most important pathogens, *Fusarium* spp. pose a threat to worldwide maize production due to their ability to cause ear and kernel rots named Fusarium ear rot (FER) and to synthesize mycotoxins in non-rotted infected kernels that can be accumulated above safety levels for humans and animals.

Many comparisons of *Fusarium* contamination and amounts of mycotoxins in organic versus conventional foods have been performed without leading to definite conclusions on the relative contamination risks. In Brazil, working with 50 organic and 50 conventional corn cobs samples, Peres et al. (2018) [2] didn't detected Aflatoxins B1, B2, G1 and G2, ochratoxin A and zearalenone, but 96% of organic corn and 100% of conventional corn contained fumonisins associated with the presence of *F. moniliforme*. The concentrations found in conventional corn were significantly higher than those of

 doi:10.20944/preprints202504.1330.v1

organic maize, but all complied with the regulation. The contamination of mycotoxins in organic maize grains at the harvest was followed over a period of three years, in organic maize from 4 farms located in 4 different Romanian counties. Fumonisins, aflatoxins and DON were detected only in two farms for one year, and at low levels [3]. Evaluating nine open-pollinated maize varieties in two farmers' fields in Galicia and in the Basque County in Spain with an experimental design under organic and conventional agriculture in 2010 and 2011, locations were the main environmental source of variation affecting fumonisins while DON was more affected by years, but there was no difference between organic and conventional environments [4]. In a previous work in Spain, both the occurrence and concentration of fumonisins B1 and B2 did not significantly differ between conventional and organic maize [5]. Most studies published in Europe in the 2000s show insignificant differences between organic and conventional production chains, or differences that are difficult to interpret. Finally, it is still very difficult to assess the cause of any differences in the relation of the farming system [6] because of the significant influence of uncontrollable factors such as the climate which is the prime factor of regulation of mycotoxin-producing fungi and mycotoxin contamination, and of the diversity of practices inside each system including the use of different varieties. However, it is interesting to monitor the situation in both production systems [7].

In contrast to the extensive research comparing organic and conventional farming, information on the fate of mycotoxins in fully integrated on-farm food chains is scarce. In the conventional agriculture system in Europe, the issue of mycotoxins is addressed by sampling, analysing and discarding contaminated batches throughout the food chain. In alternative systems, primarily organic, where farmers use self-produced seeds, store their crop at the farms before processing it themselves and sell at local markets, the opportunities for detecting contaminated kernels are non-existent. Even when the farmers are aware of the mycotoxin issues, the cost of the analysis serves as a deterrent. Consequently, there is a lack of data on the contamination levels in such an agricultural chain. This information would be invaluable to raise farmers' awareness of the risks of mycotoxin contamination and the technical solutions available to mitigate them throughout the value chain.

In the present work, samples were collected at farms after harvest, after storage for 7-9 months and after milling. All farmers cultivated landrace populations of maize, storing the crop at the farms and milling either at the farm or in a local mill. They were located in South-West of France, a region characterized by a temperate oceanic climate favourable to the colonisation of ears and kernels by *Fusarium* species. The objective was to assess the diversity of contamination levels at harvest and to track the post-harvest fate of mycotoxins.

2. Materials and Methods

2.1. Sample Collection

Samples were obtained from 13 smallholders' organic farms in Aquitaine Region, France, in the 2020 or 2021 growing seasons. All were cultivated landrace populations of maize in small-scale farming for short food circuits. Maize on the cobs was collected all along the harvest of a field for reaching 20 kg and being sent to local collectors where the cobs were shelled to obtain 5 kg of grains. Then two subsamples of 1 kg were obtained. One was transferred to Capinov (Landerneau, France), a private agricultural analysis laboratory, for multi-mycotoxins analyses. The other one was sent to our laboratory and was stored in a sample collection at 4°C. Samples were collected in the same farms after 7 to 9 months of storage in small-scale facilities, from multiple points across the batch and combined to form a representative composite sample, as done at harvest. The storage was either as cobs of maize in cribs (outdoor structures made of narrow wire mesh with a roof) or as grains in galvanized silos or big-bags. In some cases, the grains were ground at the farms in traditional mills and the meal (particle size 300-600 μm) and the flour (particle size < 212 μm) [8] were collected from different points of the batch (1 kg of each) and stored at 4°C before mycotoxin analyses.

2.2. Mycotoxin Analyses

 doi:10.20944/preprints202504.1330.v1

The provider of the mycotoxin analysis service, Capinov, has the necessary accreditations to certify the data supplied, in accordance with COFRAC standards (COFRAC accreditation n° 1-6211) valid in Europe and worldwide. The analysed mycotoxins and the limits of quantification are in Table 1. The results were delivered with an uncertainty value expressing the statistical dispersion of the values attributed to a measured quantity. Two values were significantly different when there was no overlapping of the intervals of concentrations plus and minus uncertainties.

Table 1. Mycotoxins measured in maize samples.

Mycotoxins	Method	LoQ (µg Kg^{-1})
Deoxynivalenol (DON)	LC/MS/MS	50
Nivalenol (Niv)	LC/MS/MS	50
3-acetyl Deoxynivalenol (3-ADON)	LC/MS/MS	100
15-acetyl Deoxynivalenol (15-ADON	LC/MS/MS	100
Fusarenone-X (Fx)	LC/MS/MS	50
DAS	LC/MS/MS	10
T2 toxin (T-2)	LC/MS/MS	5
HT2 toxin (HT-2)	LC/MS/MS	5
Zearalenone (ZEA)	LC/MS/MS	10
Alpha-Zearalenone	LC/MS/MS	50
Beta-Zearalenone	LC/MS/MS	50
Zearalanone	LC/MS/MS	50
Fumonisin B1 (FB1)	LC/MS/MS	20
Fumonisin B2 (FB2)	LC/MS/MS	20
Fumonisin B3 (FB3)	LC/MS/MS	20
Aflatoxin B1 (Afla-B1)	LC/Fluo	0.1
Aflatoxin B2 (Afla-B2)	LC/Fluo	0.1
Aflatoxin G1 (Afla-G1)	LC/Fluo	0.1
Aflatoxin G2 (Afla-G2)	LC/Fluo	0.1
Ochratoxin A (OTA)	LC/Fluo	0.2

2.3. Detection of Fusarium Species

DNA extraction was performed on grains, flours and meals using the NucleoMag® Plant kit (Macherey-Nagel) on 50 mg of ground samples at particle size < 0.5 mm. The process involved homogenization using a Precellys® Evolution at 6500 rpm for two 30-sec cycles (with a 5 sec break). Samples were then mixed with 500 µL of lysis Buffer MC1 and 10 µL RNase A, followed by incubation at 56 °C for 30 min. After centrifugation at 16,000 *g* for 20 min, 100 µL of clear lysate was transferred to a separation plate containing magnetic beads in Buffer MC2. Subsequent purification steps were automated using a MagMAX™ express system. The purification process included: incubation with magnetic beads for 5 min, washing with Buffer MC3 for 5 min, washing with Buffer MC4 for 5 min, washing with 80 % ethanol for 5 min, final wash with Buffer MC5 for 1 min, elution in 80 µL of Buffer MC6 for 5 min. DNA quality and quantity were assessed using 1 % agarose gel electrophoresis and UV spectrophotometry, respectively.

Quantitative PCR (qPCR) analyses were conducted to assess the abundance of *Fusarium* species. Each reaction mixture contained 20 ng of template DNA in a total volume of 10 µL, utilizing PowerTrack™ SYBR Green Master Mix (Thermo Fisher) and species-specific primers (Table 2). Amplification and detection were performed using a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™). The thermal cycling protocol consisted of an initial denaturation step at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec and annealing/extension at 60°C for 30 sec. External calibration curves generated from genomic DNA extracted from axenic cultures of *Fusarium* species (Table 2). These reference samples were also employed to validate amplification

 doi:10.20944/preprints202504.1330.v1

specificity through melting curve analysis. The data on DNA abundance of a *Fusarium* species are presented as classes of Cq ranges: 33 to 36 = +, 30 to 32 = ++, 26 to 29 = +++, lower than 26 = ++++

Table 2. Primers pairs for specific amplification of *Fusarium* species.

Primer Names	Primer Sequences	Ta 1	*Fusarium* Species	Reference
Fum1-656F Fum1-1158R	CGGTTGTTCATCATCTCTGA GCTCCCGATGTAGAGCTTCTT	60 °C	*F. verticillioides*	[9]
Fp82F Fp82R	CAA GCA AAC AGG CTC TTC ACC TGT TCC ACC TCA GTG ACA GGT T	60 °C	*F. poae*	[10]
Fg16N-F Fg16N-R	ACAGATGACAAGATTCAGGCACA TTCTTTGACATCTGTTCAACCCA	60 °C	*F. graminearum*	[10]
FC01-F FC01-R	ATGGTGAACTCGTCGTGGC CCCTTCTTACGCCAATCTCG	60 °C	*F. culmorum*	[10]
FspoF1 LanspoR1	CGCACAACGCAAACTCATC TACAAGAAGACGTGGCGATAT	60 °C	*F. sporotrichioides*	[11]

2.4. Data Analyses

Concentrations of mycotoxins in each sample are given as measured values and uncertainty intervals given by the analysis provider. Concentrations were considered different when these intervals did not overlap. Paired samples T-test analyses were made using the non-parametric Wilcoxon signed-rank test. The relationships between the different mycotoxins and the sampling times were analysed using Kendall's Tau rank correlation coefficient. The JASP (version 0.19.3; JASP Team (2024)) statistical software was used for data analysis

3. Results

3.1. Occurrence of Fusarium Mycotoxins in Maize Grains

Maize grains produced for use as food after processing were collected in south west of France (Figure 1). In the samples from 13 organic farms collected at harvest in 2020 and 2021 studied here, only one sample was not contaminated by any of the researched mycotoxins (C-64-BE-02), and another one (C-64-AR-01) was only contaminated with fumonisins at low concentrations (FB1+FB2 = 150 +/- 82 μg Kg^{-1}). In the other samples, there was a wide range of levels of contamination by DON, 15-ADON, Niv, Fx, T2, HT2, ZEA, fumonisins B1, B2, B3. The mycotoxins 3-ADON, DAS and the derivatives of ZEA were never quantifiable. At the end of storage, all the grain samples contained more than one quantifiable mycotoxin.

DON was the most prevalent mycotoxin with 10 out of 13 farm samples (77 %) having quantifiable concentrations at harvest. Half of these samples also contained quantifiable 15-ADON, but no 3-ADON was detected (Table 3). After storage, samples from two new origins were contaminated by DON, but 15-ADON became non-quantifiable in two origins previously contaminated at low concentrations (Table S1). Fumonisins were at the second place of prevalence, with 69 % samples containing quantifiable FB1 at harvest, 85 % after storage. Most also contained FB2, one third contained FB3 (Table 3).

Niv was present in 7 samples at harvest and 3 of them contained also Fx (Table 3). Niv co occurred with DON in 6 samples (60%). One sample (C-64-BU-1) contained Niv but not DON. After storage a higher number of samples contained Niv but not Fx, and the rate of co-occurrence with DON increased (83%). ZEA was present in 7/13 samples at harvest, 10/13 after storage.

Seven out 13 samples contained HT-2, and half of them also contained T-2. HT-2 co occurred with DON or Niv at harvest (Table 3). C-64-BU-1 was the only one sample positive for HT-2 but neither for DON nor ZEA. After storage, 1 new sample was contaminated with HT-2 and 3 by T-2.

 doi:10.20944/preprints202504.1330.v1

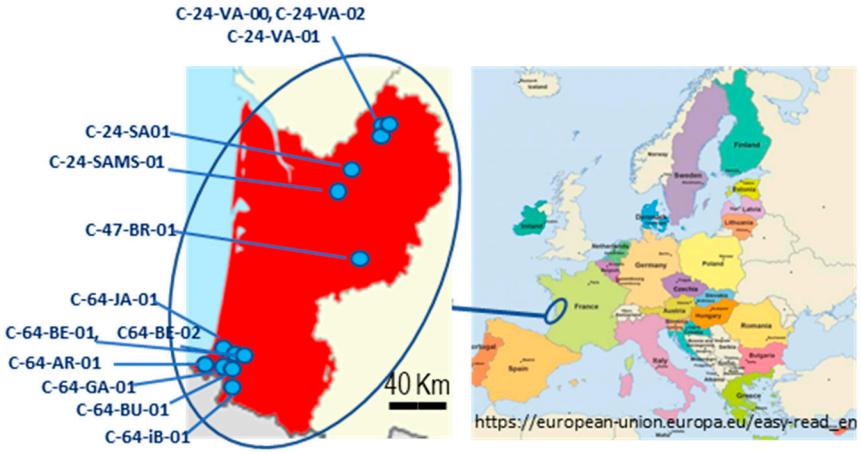

Figure 1. Sampling location in Aquitaine region (France) during 2020 or 2021 growing seasons.

Table 3. Co-occurrence of mycotoxins. Number of samples in which one or two mycotoxins were quantifiable at harvest (bold) and after storage (italics).

	DON	15-ADON	Niv	FX	ZEA	T-2	HT-2	FB1	FB2	FB3	Afla
DON	**10** - *12*	*3*	*9*	*1*	*9*	*7*	*7*	*10*	*8*	*8*	*1*
15-ADON	**5**	**5** - *3*	*3*	*0*	*3*	*3*	*3*	*3*	*3*	*3*	*0*
Niv	**6**	**3**	**7** - *10*	*0*	*9*	*6*	*6*	*8*	*7*	*7*	*1*
Fx	**3**	**2**	**3**	**3** - *1*	*1*	*1*	*1*	*1*	*1*	*1*	*0*
Zea	**7**	**4**	**4**	**3**	**7** - *10*	*7*	*7*	*8*	*7*	*7*	*1*
T-2	**3**	**1**	**4**	**2**	**3**	**4** - *7*	*7*	*7*	*7*	*7*	*1*
HT-2	**7**	**3**	**5**	**2**	**6**	**4**	**7** - *8*	*9*	*9*	*9*	*1*
FB1	**7**	**2**	**5**	**2**	**5**	**4**	**7**	**9** - *11*	*9*	*9*	*1*
FB2	**6**	**1**	**4**	**1**	**4**	**3**	**6**	**8**	**8** - *9*		*1*
FB3	**5**	**1**	**4**	**1**	**4**	**3**	**5**	**6**	**6**	**6**- *9*	*1*
Afla	**1**	**0**	**0**	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**1** - *1*

3.2. Levels of Grains Contaminations by Fusarium Mycotoxins and Fusarium Species at Harvest

At harvest, all analysed samples complied with EU regulation 2023/915 of 25 April 2023 modified by the regulation EU 2024/1022 defining a maximal level of DON concentration at 1500 µg Kg^{-1} for unprocessed maize grains (Figure 2). C-24-VA-02, the batch having the highest concentrations (1710 µg Kg^{-1}) would have been considered as conform due to the uncertainty value (504 µg Kg^{-1}) (Table S1). Except C-64-IB-01 at 1370 µg Kg^{-1}, the concentrations of the other samples were lower than or equal to 520 µg Kg^{-1} (Figure 2), which is the median value reported in a large world survey of contamination [12]. Despite a high level of occurrence observed, the contamination levels were low. ZEA concentrations were lower than 100 µg Kg^{-1} (Figure 2A), except in one sample (C-24-VA-02, 553 +/- 194 µg Kg^{-1}) which overpassed the maximum authorized level in Europe (Table 4). ZEA concentrations were positively correlated to DON concentrations (Figure 3). The concentrations of T-2+HT-2, between 12 and 58 µg Kg^{-1} (Figure 2A), were significantly lower than 100 µg Kg^{-1}, the maximum level authorized in the new EU regulation (Table 4). There was no correlation with ZEA and DON concentrations (Figure 3), these mycotoxins being produced by different *Fusarium* species.

The concentrations of FB1+FB2 in all the samples (Figure 2) were significantly lower than the maximal level of the previous and present EC regulation (Table 4), with 1,150 +/- 360 and 350 +/- 131 µg Kg^{-1} as the highest concentration for FB1 and FB2 respectively in C-47-BR-01. FB1 was always in significantly higher concentrations than FB2 while the concentrations in FB3 were always the lowest

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(Sup X). The concentrations of FB1, FB2 and FB3 were significantly correlated. Concentration in FB1+FB2 did not correlate with the other mycotoxins.

15-ADON, Niv and Fx are not regulated in Europe. In the five samples containing 15-ADON in addition to DON, the ratios of concentrations DON/15-ADON were from 0.8 to 2, except for C-24-VA-02, which had a ratio close to 10 (Table S1). The range of Niv + Fx concentration was 170 to 750 µg Kg^{-1} (Figure 2A). Pairwise Niv concentrations were lower than DON concentrations, except for the batch having the highest Niv concentration (731 µg Kg^{-1}) and a ratio DON/Niv of 0.36 (Table S1), but the correlation between the both mycotoxins was not significant (Figure 3).

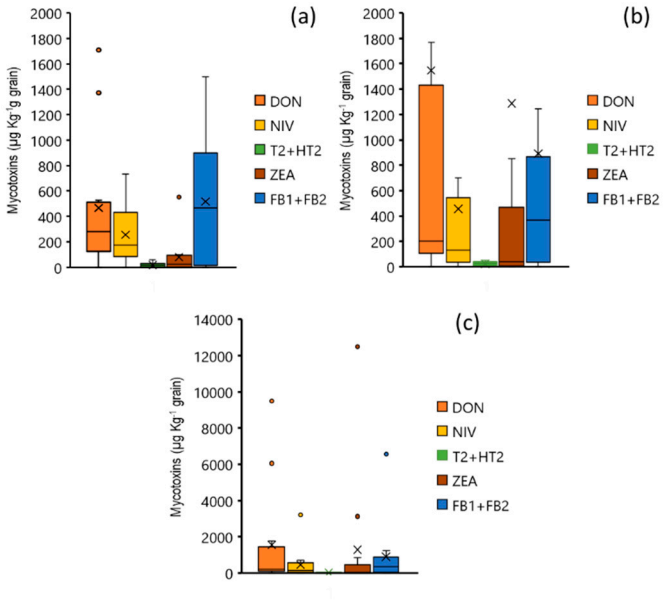

Figure 2. Box Plot of the distribution of mycotoxins concentrations in maize grains. (a): at harvest, (b): after storage with a scale limited to 2000 µg Kg^{-1}, (c): after storage.

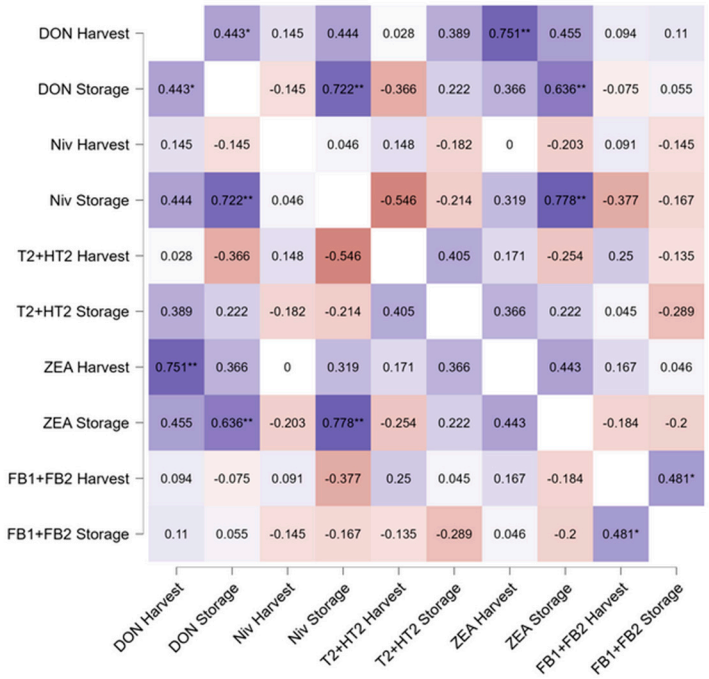

Figure 3. Correlations between mycotoxins concentrations measured in maize at harvest and after storage, Kendall's tau B heatmap. * $p < .05$, ** $p < .01$, *** $p < .001$.

 doi:10.20944/preprints202504.1330.v1

Table 4. Evolution in the European Commission regulations on maximal levels (µg Kg^{-1}) of mycotoxins in maize and some maize products.

Maize products	Mycotoxins	Commission regulation (EU) number				
		2006/1881	2007/1126	2023/915	2024/1022	2024/1038
Unprocessed grains	DON	1,750	1,750	1,750	1,500	
	ZEA	200	350	350		
	FB1+FB2	2,000	4,000	4,000		
	T-2+HT-2					100
Grains intended for direct human consumption	DON	750			750	
	ZEA	200		100		
	FB1+FB2	1,000		100		
	T-2+HT-2					50
Milling fractions of maize with at least 90 % of the particles in the milling product have a size ≤ 500 µm; flour not placed on the market for the final consumer	DON		750	750	750	
	ZEA		300	300		
	FB1+FB2		2,000	2,000		
	T-2+HT-2					20
Milling fractions of maize with Less than 90 % of the particles in the milling product have a size ≤ 500 µm; meal not placed on the market for the final consumer	DON		1,250	1,250	750	
	ZEA		200	200		
	FB1+FB2		1,400	1,400		
	T-2+HT-2					20

Overall, presence or absence of mycotoxins and correlations of concentrations might be the results of differences in colonisation by *Fusarium* species. At harvest, *F. graminearum* was detected in all the tested samples whereas *F. culmorum* was never detected. *Fusarium verticillioides* was abundant in grains of one origin and detected in another sample, the only one where *F. sporotrichioides* was detected. *Fusarium poae* was significantly observed in grains of two origins and (Table 5).

Table 5. Incidence and relative abundance of *Fusarium* species in grains and millings of maize collected in 6 farms. See Figure 1 for localisations.

Samples		*Fusarium* species				
		F. culmorum	*F. graminearum*	*F. poae*	*F. sporotrichioides*	*F. verticillioides*
C-24-VA-02	Harvest	0	++	0	0	0
	Storage	0	++++	++	++	++
	Flour	0	++++	++	++	++
	Meal	0	++++	++	++	++
C-24-VA-01	Harvest	0	++	0	0	0
	Storage	+	++++	++	0	++
	Flour	0	++++	++	++	++
	Meal	0	++++	++	++	+
C-24-VA-00	Harvest	0	++	++	++	+
	Storage	+	++++	++++	++	+

 doi:10.20944/preprints202504.1330.v1

	Flour	+	++++	++++	+	0
	Meal	+	++++	++++	++	+
C-24-SA-01	Harvest	0	++	0	0	0
	Storage	0	++	0	0	0
	Flour	0	++	++	++	+
	Meal	0	++	0	++	++
C-24-SASM-01	Harvest	0	++	0	0	+++
	Storage	0	++	++	0	+
	Flour	0	++	0	+	++
	Meal	0	++	0	0	++
C-47-BR-01	Harvest	+	+++	++	0	0
	Storage	0	+++	++	+	++
	Flour	0	+++	++	++	++++
	Meal	0	++	++	0	++

3.3. Fate of Fusarium Mycotoxins and Fusarium Species During Storage

Mycotoxin contamination can continue during storage under suitable conditions for fungi activity. After harvesting, the maize cobs were either dehulled and the grains kept in galvanised steel storage bins (C-24-SA-01) or big-bags (C-24-SASM-01; C-47-BR-01), or stored whole in narrow open-air wire mesh structures known as cribs for 8 to 9 months (all the other farms). Considering all the farms, the differences in mycotoxins concentrations at harvest and after storage was not significant at $p<0.05$ (Table 6), but significant positive correlations were observed (Figure 3).

Overall, there was a positive correlation between FB1+FB2 concentrations measured at harvest and after storage. In farms where the grains were not stored in cribs, the levels of contamination did not significantly change or decreased, except for C-47-BR-01 in which the high levels of fumonisins recorded at harvest increased dramatically during storage (x 4.38) (Table 7). This sample also exhibited the highest abundance of *F. verticillioides* (Table 5). In the farms with storage in cribs, quantifiable concentrations of FB1, and also other mycotoxins, appeared in C-64-BE-02 which was not contaminated at harvest. It is worthy to note that no increase in fumonisins was recorded in the other farms, except for appearances of both FB1, FB2, FB 3 and *F. verticillioides* at low levels in two samples which were highly affected by over-contamination with the other mycotoxins.

On overall, there was a positive correlation between DON concentrations measured at harvest and after storage ($p<0.01$). No correlation was significant for the other trichothecenes and ZEA (Figure 3). As they were at harvest, concentrations in DON and ZEA at storage were significantly correlated positively. In addition, they both were correlated with Niv concentration. In the farms where the grains were not stored in cribs, the levels of ZEA and of trichothecenes A and B did not significantly change during storage (Table 7). In the farms with storage in cribs, contrasted events were observed. Significant increases in levels of mycotoxins in grains were observed after storage in cribs, but not for all the mycotoxins and sometimes significant decreases also occurred. The levels of mycotoxins decreased from the harvest to the end of storage in C-64-BE-01 and C-64-BU-1 having low contamination levels at harvest, (Table S1) while large increases in both TCTA and TCTB were observed in 3 farms from the same locality, C-24-VA-00, C-24-VA-01, C-24-VA-02 (Table 7). With a maximum level of DON at 1500 µg Kg^{-1} for unprocessed grains in EU regulation, C-24-VA-00, and C-24-VA-02 became nonmarketable to a mill after storage. In another farm at the extreme part of the study area, C-64-IB-1, concentrations in trichothecenes and ZEA also increased while fumonisins concentrations decreased. In agreement with the changes in TCTB concentrations, an increase in abundance of *F. graminearum* and *F. poae* was noticeable for C-24-VA-00, C-24-VA-01, C-24-VA-02. *Fusarium culmorum* became detectable in two of them (Table 5). The increase was significant for Niv

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but not DON for C-64-JA-01 and C-64-IB-1. On another hand, decreases were recorded for both DON and Niv in C-64-BE-01 and C-64-GA-01. In addition, Niv concentration decreased in C64-BU-1.

The increases in TCTA concentrations were related to increased abundances of *F. sporotrichioides* and/or *F. poae*. T2 and HT2 toxins, absent at harvest in C-47-BR-01 appeared at storage (Table 7) while *F. sporotrichioides* became detectable (Table 5).

Table 6. Overall comparisons of changes from harvest to end of storage. statistical analysis with Wilcoxon signed-rank test.

Measure 1	Measure 2	W	z	p
DON Harvest	DON Storage	19.000	−1.569	0.129
Niv Harvest	Niv Storage	17.000	−1.070	0.322
T2+HT2 Harvest	T2+HT2 Storage	11.000	−1.362	0.193
ZEA Harvest	ZEA Storage	9.000	−1.886	0.067
FB1+FB2 Harvest	FB1+FB2 Storage	30.000	−0.706	0.519

Table 7. Ratios of mycotoxins concentrations at storage to concentrations at harvest.

Origin of the samples	DON+15-ADON	Niv+Fx	T-2+HT-2	ZEA	FB1+FB2+FB3
C-24-VA-02	3.36 *	3.45 *	14.28 *	1.54	S+
C-24-VA-01	2.80 *	S++	5.26 *	1.26	1.35
C-24-VA-00	12.32 *	S++	S+	490.20 *	S+
C-24-SA-01	0.34 *	0.43 *	0.42	0.67	1.52
C-24-SASM-01	1.44	nd	1.04	nd	2.92 *
C-47-BR-01	1.06	nd	S+	0.46	4.38 *
C-64-JA-01	0.84	2.60 *	S+	S+	0.86
C-64-BE-01	0.12 *	0.21 *	0	0.71	0
C64-AR-01	S+	nd	nd	nd	0.27
C-64-BE-02	S+	S+	nd	S+	nd
C-64-GA-01	0.34 *	0.27 *	nd	S+	nd
C-64-BU-1	nd	0.39 *	0	nd	0.40 *
C-64-IB-1	1.29	6.51 *	3.45 *	31.42 *	0.38 *

* Concentrations at storage and harvest are significantly different. S+= no mycotoxin detected at harvest, but significant at low concentration after storage, S++= no mycotoxin detected at harvest, but at high concentration after storage. Nd = non-detected, neither at harvest nor after storage. 0 = low concentrations at harvest and non-detected after storage.

3.4. Transfer of Mycotoxins into Milling Products

The meal and flour obtained by the farmers using dry milling processes with their own batch of grains were collected in 8 out 13 farms. For C-64-BE-1 only flour was collected. Significant positive Kendall's Tau correlations were measured between grains, meal and flour (p<0.1) for all the mycotoxins except for T-2+HT-2 having a non-significant correlation between grain concentration and flour or meal, and FB1+FB2, having non-significant correlation between grains and meal and between flour and meal (data not shown). Overall, highly contaminated grains will lead to highly contaminated mill products. We observed such a trend of higher concentrations in flours than in meals for all the mycotoxins measured. (Table 8).

Due to the uncertainty values of the measures of TCTB, differences between grains and the milling products were significant for only two origins and there was no general trend (Figure 4). Half of DON measured in grain was found after milling in flour and meal for C-24-VA-02 whereas the DON concentration in milling products of C-24-VA-01 were 2 to 3 times higher than in grain and did not change in 24-VA-00. The EU regulation 2023/915 of 25 April 2023 modified by the regulation EU

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2024/1022 defining a maximal level of DON at 750 µg Kg^{-1} (Table 4). Due to the high levels in grains, the milling products from these farms, overpassed the maximum. The increase was also significant for T-2 and HT-2 and dramatic for ZEA (Figure 4) in 24-VA-01, whereas for C-24-VA-00 TCTA concentrations decreased in milling products and ZEA in meal.

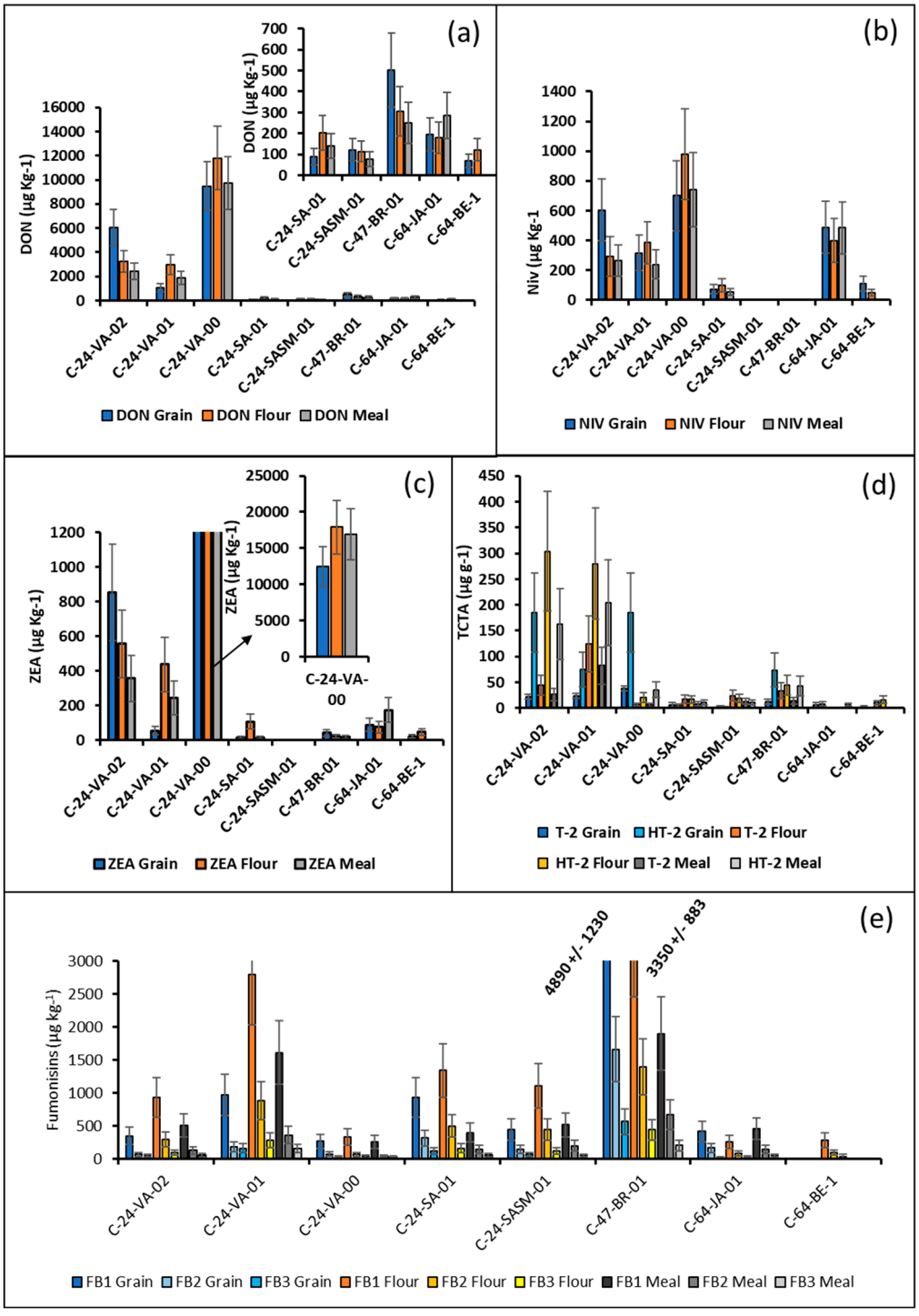

Figure 4. Mycotoxins concentrations measured in grains after storage and milling products obtained on 8 farms. (a): DON, (b): Niv, (c): ZEA, (d): T-2 and HT-2, (e): FB1, FB2 and FB3. Error bars are +/- uncertainties.

Table 8. Ratios of mycotoxins concentrations in flour to concentrations in meal.

Ratio	Mycotoxins							
Flour/meal	DON	Niv	T-2	HT-2	ZEA	FB1	FB2	FB3

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C-24-VA-02	1.35	2.06	1.63	1.87	1.56	1.82	2.31	1.59
C-24-VA-01	1.60	0.82	1.50	1.37	1.79	1.73	2.45	1.83
C-24-VA-00	1.21	0.72	1.13	0.59	1.06	1.26	1.44	1.43
C-24-SA-01	1.44	0.74	2.12	1.60	6.56	3.35	3.53	3.05
C-24-SASM-01	1.48	/	1.91	1.65	/	2.15	2.24	2.35
C-47-BR-01	1.22	/	2.36	1.04	1.37	1.76	2.11	2.15
C-64-JA-01	0.63	1.22	/	0.00	0.43	0.56	0.55	0.68

/ indicates non-quantifiable mycotoxin both in flour and meal.

The fumonisins concentrations in flour samples were all lower or equal to the concentrations in the corresponding grains, and lower or equal concentrations were measured in meal than in flour (Figure 7). However, the milling products for C-47-BR-01 still largely overpassed the EU maximum level which is 1000 μg Kg^{-1}. Due to the lower maximum for milling products than raw grains, the flours from C-24-01 and C24-SASM-01, as well as both flour and meal for C-24-VA-01, did not comply with the EU rule while the grain did. Consequently, these products were not marketable as food.

3.5. Ochratoxin A and Aflatoxins in Maize Grains and Millings

In the samples analysed in this study, there was a wide range of levels of contamination by DON, 15-ADON, Niv, Fx, T2, HT2, ZEA, fumonisins B1, B2, B3, but the presence of OTA and Aflatoxins was scarce. The typical storage mycotoxin, OTA, was found in only one sample, C-24-VA-02 after storage at low concentrations, 0.58 (+/-0.26) μg Kg^{-1} in grains, 1.2 (+/- 0.40) in flour, 0.74 (+/- 0.33). Pre-harvest contamination by Aflatoxins was found in only one sample (C-64-JA-01) at a low concentration, 0.39 +/-0.17 μg Kg^{-1} for the sum Afla-B1, Afla-B2, Afla-G1, Afla-G2. Post-harvest we found these mycotoxins in the three farms where grains were not stored in cribs. They were at low concentrations, 0.47 (+/- 0.21) and 0.87 (0.38) μg Kg^{-1} for the sum of aflatoxins in flours of C-24-SA-01 and C-24-SASM-01 respectively, but not in the meals. In both samples, aflatoxins were not quantifiable in unprocessed grains. In C-47-BR-01, grains, flour and meal were dramatically contaminated by Afla-B1, and Afla-G1, without differences between the fractions, and contained also Afla-B2, and Afla-G2 (Table 9).

Table 9. Concentrations of aflatoxins developed in storage at the farm C-47-BR-01, and transferred into milling products. In μg Kg^{-1}. Values in brackets are uncertainties.

	Afla-B1	Afla-B2	Afla-G1	Afla-G2	Sum
Grain at harvest	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Grain after storage	99.6 (+/- 43.8)	5.5 (+/- 2.4)	70.0 (+/- 30.8)	4.0 (+/- 1.7)	179 (+/- 74)
Flour	80.1 (+/- 35.3)	4.1 (+/-1.8)	38.2 (+/-16.8)	2.5 (+/-1.1)	125 (+/- 55)
Meal	134 (+/- 58)	5.8 (+/- 2.5)	54.5 (+/- 24.0)	3.1 (+/- 1.4)	197 (+/-81)

4. Discussion

4.1. Maize Grains Contamination by Mycotoxins and Fusarium Species

In the maize grain samples collected at harvest and after storage we analysed in this study, there was a large diversity of occurrence and a wide range of levels of contamination by DON, 15-ADON, Niv, Fx, T2, HT2, ZEA, Fumonisins B1, B2, B3. The presence of OTA and Aflatoxins was scarce.

Fumonisins have been widely reported as the prevalent mycotoxins in maize grain in southern Europe [13], and in the large international survey reported by Gruber-Dorninger et al. 2019 [12], 80 % of the samples contained fumonisins. A lesser percentage of samples were contaminated with fumonisins and the concentrations were lower than those reported in Mediterranean areas (Figure 2). FB1 and FB2 were present in Algerian samples in a concentration range of 289–48,878 μg Kg^{-1} [19].

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In Spain, the observed range of concentrations was 26.0–63,100 with a mean of 5,734 µg Kg^{-1}, 20 % exceeding the EU limit [20].

We observed DON as the prevalent mycotoxin with a percentage similar to those reported from North America, and a high level of co-contamination with 15-DON at concentrations from 50 % to 125 % that of DON. These ratios were higher than those reported by Oliviera et al. (2017), where 15-ADON contributed, on average, to 31% of the total contamination by DON and its derivatives [18]. In a 7-year survey of corn grain in the United States (711 samples), occurrences were 76 % DON and 48 % 15-ADON, but also 14% 3-ADON [14]. In Michigan harvested maize grain 93 to 100 % contained DON, 47 to 100 % contained 15ADON [15]. However, the observed DON and its derivative rate is higher than in most of the published works from various world regions. The frequency of DON contamination found in some Brazilian studies was lower than 9 % [16,17] but reached 48 % in a survey of maize samples from the south region of Brazil [18]. In South Europe and the Mediterranean Basin, lower rates of DON occurrence were also recorded in some studies but not in others. On a total of 20 maize samples destined for human consumption purchased from local markets in the western region of Algeria, 13 (43 %) were positive for DON [19]. Studying 98 samples of maize kernels without visible signs of mould growth collected in the years 2015–2019 in 26 grain stores located in different Spanish regions, DON was quantified in 31 samples, and only 5 samples had quantifiable levels of 3-ADON+15-ADON [20]. In northern Italy, Emilia Romagna region, in 2014, DON was detected in 59% of the analysed samples from 51 farms whereas it is generally rare in Italian samples. The year 2014 was reported as alarming for DON contamination in Europe [21]. 65-100 % of the samples of a large survey of maize grain samples collected from 88 storage centres in Northern Italy contained DON [13]. In a global survey program to monitor mycotoxin contamination of feed raw materials collected from 2008 to 2017, 67 % of 12600 maize samples were contaminated with DON [12]. However, this large survey was based on samples voluntarily sent by farmers or feed industry for analyses, which could be those suspected to be at risk.

Co-occurrence of mycotoxins is a common observation in maize grains with values ranging from 60 to 90% of positive samples with more than one mycotoxin. Co-occurrence of Niv plus its derivative Fx, with DON were observed very often [22]. Here, the rates of positive samples for Niv were also high compared to published works. A Brazilian study reported only 10 % and 2.5 % Niv and Fx respectively [17], while 76% of the corn samples from the south region of Brazil were contaminated with Niv [18]. In samples analysed in the United States, neither Niv nor F-x were found [15] or Niv, but no F-x was found at a rate of 7 % [14]. This survey of published work shows the diversity of levels of occurrence of DON and its derivatives. The data collected from the grains of landrace maize grains from organic agriculture stored at small farms in southwest of France tends to highlight a high level of occurrence of TCTB, but at low concentrations (< 550 µg Kg^{-1}) in most of the samples and no one exceeded the EU limit. However, there is a concern to consider DON derivatives and Niv plus Fx in the authorized maximal concentrations of TCTB.

As for TCTB, there was a high rate of samples contaminated with HT-2, but not T-2 which had not been reported previously, but low concentrations. Fusilier et al. (2022) found 19 and 4 % of T-2 and HT-2 respectively in their samples [13]. In maize samples from Algerian markets, 100 % of samples contained T-2 with a mean concentration of 25 µg Kg^{-1} whereas HT-2 was never found [19]. In Spain, only 5 % of the samples analysed contained T-2 and HT-2 with medians lower than 12 µg Kg^{-1} [20]. Those toxins were not detected by Tonial Simões et al. (2023) in a field experiment on maize varieties [17]. The rate of positive samples recorded in the USA over 7 years were 7.5% and 5.9% with a median at 10 and 38 µg Kg^{-1} for T-2 and HT-2 toxins respectively [14]. 12% of maize analysed worldwide showed detectable levels of T-2 and HT-2 [12].

The occurrence of ZEA and concentrations were in ranges reported in the literature. ZEA was detected in 44 % of the samples in the large survey reported by Gruber-Dorninger et al. (2019) [12]. 42 % were reported in the south of Brazil [18], whereas 69% of positive samples were found in Michigan [15] and 25 to 91 % in Italy [13]. Values lower than 23 % of positive samples were recorded in the USA [14], Algeria [19], and Spain [20]. In these studies, median values of 115 µg Kg^{-1} (mean

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302) [14], median 110 µg Kg^{-1} (mean 837) [20] or mean 109 µg Kg^{-1} [19] were recorded. In Northern Italy low concentrations with means lower than 25 µg Kg^{-1} were measured most years but in 2014, the mean concentration was 364 µg Kg^{-1} [13]. ZEA always co-occurred with DON, with significant correlations between the concentrations. In maize intended for feed, DON and ZEA concentrations were positively correlated [12]. Both mycotoxins are known to be produced by the same species of *Fusarium*.

Most notable mycotoxigenic *Fusarium* species are members of the *Fusarium sambucinum* and *Fusarium fujikuroi* species complexes [23]. We used specific primers for assessing the presence and relative abundance of five *Fusarium* species in some maize samples being all contaminated with DON at different concentrations: *Fusarium verticillioides* as a representative member of the *Fusarium fujikuroi* Species Complex producing fumonisins and four members of the *Fusarium sambucinum* Species Complex: *F. culmorum* and *F graminearum* known to produce DON, ZEA and Niv (some strains only), *F. poae* producing Niv and T-2/HT2, and *F. sporotrichioides* producing T-2/HT-2 and ZEA [23]. The prevalence and distribution of the *Fusarium* species depends on climatic conditions and agronomic practices., *F. graminearum* tends to be the main species present in maize in cooler temperate regions while *Fusarium fujikuroi* complex species (*F. verticillioides*, *F. subglutinans*, and *F. proliferatum*) dominate in the warmer temperate regions [24] such as Tunisia [25]. *Fusarium culmorum* is known to occur rarely in maize ears, but it is highly aggressive and it functions better in cooler seasons [26]. Our data reflects a situation of temperate region with a low incidence of *F. verticillioides* contrasting to data from warmer regions [25] and absence of *F. culmorum*. In a previous study in France the 15-ADON trichothecene chemotype predominated in 85.4 % of the 185 *F. graminearum* isolates from maize whereas the NIV chemotype was found in 14.6 % [27]. According to the recorded occurrence of mycotoxins in the present study, the putative predominant chemotype was DON/15ADON but Niv/Fx was also well represented. This and co-occurrences with HT-2 indicated contaminations by different *F. graminearum* strains and contaminations by both *F. graminearum* and *F. poae*. *Fusarium sporotrichioides* was only detected in C-24-SA-01 in agreement with the contamination in T-2, but not HT-2. Actually some *F. graminearum* strains could also produce HT-2 [28]. It is known that the conditions required for fungal growth are not necessarily the same for mycotoxin production consequently the presence of a mycotoxigenic *Fusarium* species does not necessarily imply mycotoxin production [29]. The levels of contamination in mycotoxins and the presence of potential producers did not match most of the time. Neither *F. culmorum* nor *F. poae* were detected in C-24-VA-02 contaminated with Niv at 175 µg Kg^{-1} whereas *F. poae* was detected in C-47-BR-01 which did not contain Niv. A strain of *F. graminearum* with appropriate chemotype could have produced Niv and a *Fusarium* species can be present on grains without having produced mycotoxins. Surprisingly, *F. verticillioides* was not detected in this sample having the highest concentrations in fumonisins whereas it was at a high level in C-24-SAMS-01 containing 7 times less FB1.

In Europe, pre-harvest aflatoxin contaminations of maize are predicted to increase in the course of climate change [30], but aflatoxins are still emerging in France. Analysing more than 500 maize samples from France taken at harvest during 2018-2020, Bailly et al. (2024) observed that only 7% were contaminated with mean levels of 3.8 µg/kg and 5.9 µg/kg for Afla-B1 and the sum of aflatoxins [31]. Actually, an aflatoxin risk index in the area covered by the present study was estimated as very low with the present climate, in agreement with only one pre-harvest contamination at a very low concentration we observed, but it is expected to increase and become high in the intermediate 2050 climate scenario [32].

4.2. Fate of Mycotoxin at Storage and During Dry Milling

An objective of the study was to compare the contaminations in a same batch of maize before and after storage under farm conditions. Despite precautions in the sampling strategy, heterogeneity due to on farm practices have to be considered. However, different trends in changes of mycotoxin concentrations were observed. Drying and storage in cribs as cobs is an ancient practice preserved in

 doi:10.20944/preprints202504.1330.v1

this part of France as a part of a local traditional way to produce a local population maize which was supposed to affect the levels of contaminations. This hypothesis was tested.

For fumonisins and *F. verticillioides*, the only case of dramatic contaminations during storage was for grains stored in big-bags as a result of default of drying. This batch was also dramatically contaminated with aflatoxins. The use of cribs did not appear as a risk factor. Changes in amounts of fumonisins during storage in silos has been reported by several authors with sometime increases [24], sometime decreases [33,34]. In a Brazilian region characterized by humid tropical weather with hot and rainy summers and dry winters, the maize grains were put in 60 kg jute sacks (5 sacks per hybrid), stacked over wooden boards and stored for 12 months in a well-ventilated warehouse, located near the production area [34]. The authors related a contamination of 90 % with fumonisin B1 in a range of concentrations 0.9 to 49 μg g^{-1}, and 97 % with fumonisin B2 in a range of concentrations 2 to 29 μg g^{-1}. After 140 days of storage, a tendency towards decreased FB1 mean concentrations and an overall decrease in the number of CFU/g for *Fusarium* spp was observed, although FB1 levels showed some variability [34]. However, in most farms storing maize in cribs in Brazil studied by Quieroz et al. (2012), the total fumonisin levels did not increase throughout the storage period, except in one farm [35]. In traditional storage structures constructed using mud and wattle commonly fitted with a grass thatched roofs used in Uganda, fumonisins also decreased after 4 months of storage from an average of 5,700 to 2,800 μg kg^{-1} both shelled or as cobs [36]. Maize stored in ventilated structures close to the case in cribs had significantly lower fumonisin levels than maize stored in non-ventilated structures for 6 months. In Benin, a decreasing trend in fumonisin levels detected in maize samples throughout the storage time was observed, but it was not significant in all seasons [37]. At the opposite, fumonisins in grains < 15% humidity increased by 3 to 45 % after 6 months in different kind of bags used for storage under local environmental conditions in Kenya with a trend to higher rates in dry than in wet treatment [38]. Studying the evolution of mycotoxins during grains storage in bags, Gaël et al. (2020) observed that fumonisins increased slowly until month 10 and significantly from 10 to 18 months [39]. This progression was slowed down when plant biopesticides were added as dried leaves. In Mediterranean Europe, Spain, the accumulation of FB1 decreased by 63%, after three months of storage [33]. In Portugal, fumonisins showed a tendency to increase (20 to 40%) during six months of storage but no correlation between the levels of fumonisins and the climatic parameters recorded in experimental silos was established, even if meanwhile temperature and CO_2 levels increased [24]. On overall, in traditional maize storages including cribs, the risk of fumonisins over contamination is limited.

Contrary to the fate of fumonisins, trichothecenes A and B and ZEA were affected only in some farms using cribs. They tended to decrease when contaminations at harvest were low and to increase significantly for the highest contaminated batches, with associated new developments of Fusarium species known to produce each kind of mycotoxins. In contrast to the lack of correspondence between *Fusarium* species and mycotoxins previously observed on single samples at a given time, changes during storage in detected *Fusarium* species and their abundance were linked to increases in mycotoxin concentrations. There are not many studies on trichothecenes concentration changes in maize grains during storage. In a laboratory experiment, Venslovas et al. (2022) observed decreases about two times of DON concentration in dried maize (up to 7% moisture content) after 6 months of storage at 12°C and 20°C while at 4°C after 3 months of storage it also decreased, and then after 6 months it increased to the same concentration as at the beginning of the experiment [40]. DON and T-2 absent at harvest were not found after storage [24]. Present and previous data do not allow to conclude on a general trend in fate of TCTB in storage, but it is clear that under unfavourable conditions dramatic over-contaminations may occur, rendering batches of grain unmarketable and dangerous to human and animal health.

ZEA increased dramatically in two batches stored in cribs, overpassing the EU maximum level (350 μg Kg^{-1}) and the maximum (611 μg Kg^{-1}) found in a survey of the literature in Europe [41], and appeared in two other samples. During the storage period of 6 months in cribs in 10 Brazilian farms, there was no difference between zearalenone levels in samples collected at 4 different times from 6

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farms. However, in 4 farms there was a wide variation in these levels with sometimes decreases. The authors attributed the wide range from one sampling period to another, to the difficulty of obtaining homogeneous samples in this type of storage (ears with husk) [35]. In hermetic bags, ZEA started to increase significantly after 10 months of storage and increased by 6 times in the next following months [39]. The risk of maize contamination with ZEA during storage period is a cause for concern.

expected under a random association in a 7-year survey with more than 700 grain samples in the USA [14]. Recently, in Brazil aflatoxin-fumonisin co-exposure occurred in 8.33% of the samples from organic farming [45], whereas previously co-occurrences between 33 and 54% had been recorded in 24 samples of maize commercialized in São Paulo [18], and between 10 to 45 % in the southern region of Brazil [46].

There are relatively few studies on AFs in dry-milling of maize. Researchers observed a reduction in AFB1 content in the endosperm fraction, although this reduction was not statistically significant [43]. In the highly contaminated farm at storage, the contamination of Aflatoxins could not be reduced during processing. The concentrations were 30 times higher than the EU maximum authorized value in EU (4 µg Kg^{-1}) and 2.5 the maximum previously recorded in French pre-harvest samples [31]. In northern Italy two out 500 maize samples collected over 1995–1999 from storage bins and feed mills within 3 months after harvest had Afla-B1 levels of as much as 109 to 158 µg Kg^{-1} [47]. In about 300 to 400 samples collected each year from 2011 to 2021 in 88 storage centres the maximum of Afla-B1 concertation was 145 g Kg^{-1} [13].

5. Conclusions

Our data reflects a situation of temperate region with a low incidence of *F. verticillioides* contrasting to data from warmer regions, and absence of *F. culmorum*. The data collected from the grains of landrace maize grains from organic agriculture stored at small farms in southwest of France tends to highlight a high level of occurrence of mycotoxins, but at low concentrations in most of the samples and no one exceeded the EU limit. Except for ZEA. Reported mycotoxin concentrations at harvest complied with EU thresholds when limits are set by EU regulations. In the two seasons analysis study in South-West France on organic maize grains, DON was the most prevalent mycotoxin at harvest (more than 3 out of 4 samples were contaminated) then come fumonisin contamination for more than two third of the samples.

Fumonisins and ZEA contamination frequency tended to raise during storage. Anyhow, concentrations evolved during storage without a strong statistical link between harvest and end of storage, but globally more diverse mycotoxins were found after storage and some mycotoxins were found more frequently associated in the same samples after storage (e. g. ZEA and DON, ZEA and Niv, DON and Niv). This study led us to characterize traditional smallholder's agronomic practices and estimate their impact on occurrence and level of mycotoxins. For example, the use of cribs that did not appear as a risk factor, except in few samples. OTA and Aflatoxins which are known to be typical storage mycotoxins have been found in only one sample from cribs but have been found in all the three samples not stored in cribs.

The prevalence and distribution of the *Fusarium* species depends on climatic conditions and agronomic practices in field. The low number of samples did allow to unravel climatic and agronomic factors lowering mycotoxin risks, but recommendations were given individually to the participating farmers. Presence or absence of mycotoxins and correlations of concentrations might be the results of differences in colonisation by *Fusarium* species during production step and then during storage. It is still very difficult to assess the cause of any differences in the relation of the farming system because of the significant influence of uncontrollable factors such as the climate which is the prime factor of regulation of mycotoxin-producing fungi and mycotoxin contamination, and of the diversity of practices inside each system. As an example, the position of the crib in the farm landscape was suspected to explain the high levels of over-contaminations in a specific case.

Milling process of maize grains did not change dramatically mycotoxin concentrations in flour or meal (except for T-2+HT2 and FB1+FB2 toxins). As a consequence, grain batches with

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concentrations higher than the UE regulation led to milling products non-suitable for trade. However, this study confirms that a high level of contamination at harvest and after storage leads to contaminated milling products but not necessarily with the same fungi and thus mycotoxins. It highlights the need to monitor the contamination at production step but also to continue monitoring the risk during storage (especially when moisture cannot be removed). Processing is not an option to count on to deliver safe products within the legal limits.

The data acquired in this work will be used to raise awareness of the mycotoxin problem among farmers producing, storing and processing maize on-farm, and to assist them in their efforts to improve the safety of their products.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Table S1: Mycotoxin concentrations in all the maize samples analysed.

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