Fungal species and multiple mycotoxin contamination of cultivated grasses and legumes crops

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The quality of grasses and legumes crops used for animal feed for the combined determination of fungal species and mycotoxin measurements were explored in samples collected from the fields of stock-farms located in North-western part of Russia. The occurrence of aflatoxin B_1, alternariol, citrinin, cyclopiazonic and mycophenolic acids, deoxynivalenol, diacetoxyscirpenol, emodin, ergot alkaloids, fumonisins, ochratoxin A, PR-toxin, roridin A, sterigmatocystin, T-2 toxin and zearalenone, were determined using enzyme-linked immunosorbent assay. The most common fungi were *Cladosporium,* followed by *Phoma*, *Alternaria*, *Aureobasidium*, *Acremonium* and *Fusarium*. Invariably high incidence of alternariol, cyclopiazonic acid, emodin and ergot alkaloids was detected in all forage types. The contribution of the fusariotoxin contamination appeared to be less significant. The grasses and grass-legume mixtures before the first cut for the year were similar in terms of high incidence of sterigmatocystin. The plants were allowed to regrow, and the complex of four regularly occurring components was supplemented with fumonisins, mycophenolic acid, and ochratoxin A.

Key words: forages, grasses, legumes, mycotoxins, fungi

Introduction

In the recent years, there have been a growing number of publications on the multiple mycotoxin contaminations of forages (Binder et al. 2007, Cheli et al. 2013, Driehuis 2013). The forage crops constitute the major feed component in the diet of domestic animals, making them the basis for the agricultural and rural economies. In many countries, a considerable share of areas of the agricultural lands is under cultivation of forage crops. The growth of commonly occurring filamentous fungi in forages may result in the production of mycotoxins, which can cause a variety of negative implications on animal health and welfare.

The major mycotoxins in commodities reported to date includes aflatoxin B_1 (AB_1) produced by *Aspergillus* species, ochratoxin A (OA) produced by *Penicillium* species, and fusariotoxins (deoxynivalenol [DON], diacetoxyscirpenol [DAS], fumonisins [FUM], T-2 toxin [T-2], and zearalenone[ZEN]) produced by *Fusarium* species (Frisvad et al. 2006).

However, at present it has been shown that the fungi are capable of producing hundreds of toxic chemicals, most of which are not included in the routine analyses. Production of many other types of toxic metabolites forms fungi belonging to these genera has been reported. *Penicillium* spp. produce mycophenolic acid (MPA) and PR-toxin (PR) (Wei et al. 1975). Many *Penicillium* and *Aspergillus* species are known to produce cyclopiazonic acid (CPA) (Cole and Cox 1981, Weidenbörner 2001) and sterigmatocystin (STE) (Burdock and Flamm 2000, Engelhart et al. 2002). Citrinin (CIT) is a mycotoxin produced by several species of the genera *Aspergillus*, *Penicillium* and *Monascus* (European Commission 2012). Other commonly occurring compounds include alternariol (AOL), a metabolite produced by many *Alternaria* species, mainly, *A. alternata* (Zajkowski et al. 1991). Emodin (EMO) biosynthesis has been described for several fungi belonging mainly to the genera *Penicillium*, *Aspergillus*, *Phoma*, *Cladosporium,* and *Trichoderma* (Cole and Cox 1981, Gessler et al. 2013). Macrocyclic trichothecene roridin A (RoA), is produced by cellulolytic fungi of the genus *Myrothecium*. *Claviceps* fungi are the major source of ergot alkaloids (EA).

However, the true dimensions of problems related to the animal health after using the mycotoxins contaminated feed are still unknown. One of the reasons might be the co-occurrence of more than one mycotoxin in the feed and their interaction that may possibly result in different effects on the animals, which are so far not given importance.

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Mycotoxin contaminations of herbaceous plants may occur in the field during the vegetation or harvesting and processing, as well as in the course of further storage that is accompanied by drastic changes in aeration, temperature, and biochemical composition. In addition to the frequent loss of sealing, the stored hay with elevated moisture level, when fed in portions, offers enough possibilities for active fungal growth, including those capable of mycotoxin production. To assess the effects of these technological processes in forage contamination, it was necessary to have information on the mycological and mycotoxicological state of the cultivated plants from the moment of their mowing. Unfortunately, there is little information available on this matter (Garon et al. 2006, Mansfield et al. 2008, Skladanka et al. 2013).

The present study aimed at a combined study of the mycological composition and mycotoxin content in the types of grass- and legume-based forages with special attention on the time of their mowing.

Materials and methods

The samples of the plant material were collected in 2014 from the fields of stock-farms located in five districts of North-western part of Russia (Leningrad region). The plant samples were picked up before the first mowing (end of May to the beginning of June, 29 samples from the different fields) and before the second one (end of July to the beginning of August, 15 samples from the fields). Collections included three forage types: grasses (type I), mixture of clover *(Trifolium pratense* L.*)* and grasses (type II), and alfalfa (*Medicago sativa* L.) mixed with timothy (*Phleum pratense* L.) (type III). The grasses were represented by fescue (the grass family Poaceae), festulolium (the hybrid cross between the *Festuca* and *Lolium* species), cock's-foot (*Dactylis glomerata* L.), timothy and ryegrass (*Lolium* spp.). The samples were collected from the agricultural fields infested with weeds, such as dandelion (*Taraxacum* spp.), buttercup (*Ranunculus* spp.), chamomile (*Matricaria* spp.), common thistle (*Cirsium arvense* L.), knotweed (*Polygonum* spp.), and wild violet (*Viola* spp.).

All samples were collected from several (at least ten) places of a field by cutting, on an area 30 × 30 cm, the aboveground parts of all plants at a distance of 2–3 cm from the soil surface. Samples of plant mixtures, as well as individual clover and alfalfa plants, were taken from the same fields. The freshly cut plants were chopped with sterile scissors into 1–2 cm lengths and thoroughly mixed.

For mycological analysis, 10 g of a prepared plant material was put into a conical flask, 90 ml of sterile distilled water was added, and the flasks were kept in a shaker at 120 rpm for 30 minutes. Then the content of the flasks was filtered through a capron screen in sterile conditions, and the obtained suspension was used to prepare a ten-fold dilution series.

The potato sucrose agar (PSA) containing Triton X-100 (4 µl l^{-1}) and streptomycin (300 mg l^{-1}) was poured into 90-mm Petri dishes and kept at room temperature to set. To every dish 5 µl of the suspension was added and evenly distributed over the surface with a glass spatula and then placed into an incubator at 23°C. At least 10 Petri dishes were used per sample. After 8–10 days of incubation, the number of colony forming units (CFU) per plate was counted, and total CFU per sample was also calculated. Then isolated fungi were grown in pure culture and were identified with the help of standard manuals based on the morphological and growth characteristics (Ellis 1971, Gerlach and Nirenberg 1982, Samson et al. 2002). The results of mycological analysis were presented as incidence, i.e., the ratio of the number of samples in which the taxon was detected (n+) to the total number of analysed samples (n), and quantity, i.e., the number of CFU in 1 g of plant tissue (CFU g^{-1}) for the positive samples of forage types.

For the mycotoxicological analysis, 100 g of the material chopped with scissors were kept in a heating chamber with convection at 50°C for 1 hour, followed by grinding in a laboratory mill. A mixture of acetonitrile and water (84:16, v/v) in the ratio of 1:10, w/v was added to the weighed portions, and they were intensively shaken twice, at the beginning and end of 14–16 hours of stationary extraction. Mycotoxins, such as aflatoxin AB_1, AOL, CIT, CPA, DON, DAS, EMO, EA, FUM, MPA, OA, PR, RoA, STE, T-2, and ZEN were determined in the extracts using the qualified test systems for enzyme-linked immunosorbent assay (ELISA) as described in the study (Kononenko et al. 2012). The accuracy of determinations (102±4%) in grasses and legume plants was evaluated at reference samples spiked with mycotoxins in accordance with the generally accepted procedure (Kononenko et al. 1999). On an average, the relative standard deviation was 11 or 16% under repeatability or reproducibility conditions, respectively. In some cases, ELISA results were confirmed by liquid chromatography–mass spectrometry (LC-MS/MS) method (Komarov et al. 2008). The amounts of mycotoxin (min, mean, and maximum, µg kg^{-1}) were calculated, if the number of positive samples (n+) was greater than three, and if they were smaller, the initial values of concentrations were shown. The data were subjected to non-parametric one-way ANOVA (STATISTICA 10.0). The significance was declared at $p < 0.05$.

Results

Mycological studies had shown that before the first cut all plants had mycobiota consisting of representatives of six genera *Cladosporium, Phoma*, *Alternaria*, *Aureobasidium*, *Acremonium,* and *Fusarium* (Table 1). The occurrence of fungi from the other five genera, Mucoraceae family as well as *Trichoderma viride* Pers. was not so regular. A total number of micromycetes in all types of grass- or legume-based forages was quite comparable. The fungi of genus *Cladosporium* (*C. cladosporioide* (Fresen.) G.A. de Vries, *Cladosporium* spp.) was found to prevail in the plants. There were slightly fewer fungi of genera *Phoma* and *Alternaria* (*A. alternata* [Fr.] Keissl., *A. tenuissima* [Kunze] Wiltshire, *Alternaria* spp.), followed by *Aureobasidium (A. pullulans* [de Bary & Löwenthal] G. Arnaud) and *Fusarium*. The fungi of the last genus were found in 13 of 29 analyzed samples and belonged to nine species. The intensity of *Fusarium* contamination was higher in clover-grass mixtures (type II) than in grasses (type I) and in alfalfa–timothy mixtures (type III). *F. avenaceum* (Fr.) Sacc., *F. anguioides* Sherb., *F. sporotrichioides* Sherb., and *F. tricinctum* Corda Sacc., occurred in all types of grass- or legume-based forage while *F. semitectum* Berk. and Ravenel and *F. culmorum* (W. G. Smith) Sacc. were detected in types I and II. The rest species, *F. equiseti* (Corda) Sacc., *F. proliferatum* (Matsush.) Nirenberg and *F. poae* (Peck.) Wollenw. in quantities of 33, 67 and 83 CFU g^{-1} were found only in the clover-grass mixtures.

Table 1. The mycobiota of different forage types before the first mowing

Taxon	type I (n = 9)		type II (n = 15)		type III (n = 5)	
	n^+	CFU g^{-1}	n^+	CFU g^{-1}	n^+	CFU g^{-1}
Acremonium spp.	2	489	5	1413	0	0
Alternaria spp.	6	6800	13	3533	4	7360
Aspergillus spp.	1	44	0	0	0	0
Aureobasidium sp.	4	756	3	240	1	160
Cladosporium spp.	8	25689	13	15067	5	18820
Fusarium spp.	2	444	9	1600	2	720
Gliocladium spp.	0	0	1	160	0	0
Mucoraceae	2	444	0	0	0	0
Mycelia sterilia	4	1200	10	4907	4	960
Penicillium spp.	4	222	1	53	1	80
Phoma spp.	8	7022	13	7147	5	8480
Scopulariopsis sp.	0	0	1	53	1	160
Trichoderma viride	1	44	0	0	0	0
Other fungi	1	133	6	560	3	880
Totally		43287		34733		37620

Type I = grasses; type II = clover-grass mixtures; and type III = alfalfa-timothy mixtures; n = total number of analyzed samples, n^+ = the number of samples where a taxon is found.

Both the incidence and quantity of fungi belonging to genus *Penicillium* (*P. brevicompactum* Dierckx, *P. roqueforti* Thom and *Penicillium* spp.) were low. Other taxa, such as *T. viride*, *Aspergillus* spp., fungi of Mucoraceae family were found solely in grasses (type I); and *Gliocladium* spp. was detected only in clover-grass mixtures (type II). Fungi *Acremonium* spp. occurred only in forage types I and II, and at the same time *Scopulariopsis* sp. was detected in types II and III. Mycelia sterilia fungi that did not form spores and the group of non-identified fungi were found in all forage types.

From the results shown in Figure 1, it can be concluded that multiple mycotoxin contamination is present in forages of types I and II. They were similar in terms of high incidence of AOL, CPA, EMO, EA, and STE. However, clear differences were noted. The clover-grass mixtures were contaminated with greater amounts of AOL and EMO. Many mycotoxins also occurred in type II forage more often, especially OA, MPA, PR, T-2, DAS, FUM, and RoA.

The presence of AOL, CPA, EMO, and EA was found in the alfalfa-timothy mixture (type III). The amounts of AOL and EMO detected in this type of forage were similar to that of grasses. The fact that MPA was common, while T-2, DAS, and OA occurred slightly more rarely, brought alfalfa-timothy mixtures were close to clover-grass mixtures type. In terms of finding PR in one sample and missing of FUM brought them close to type I.

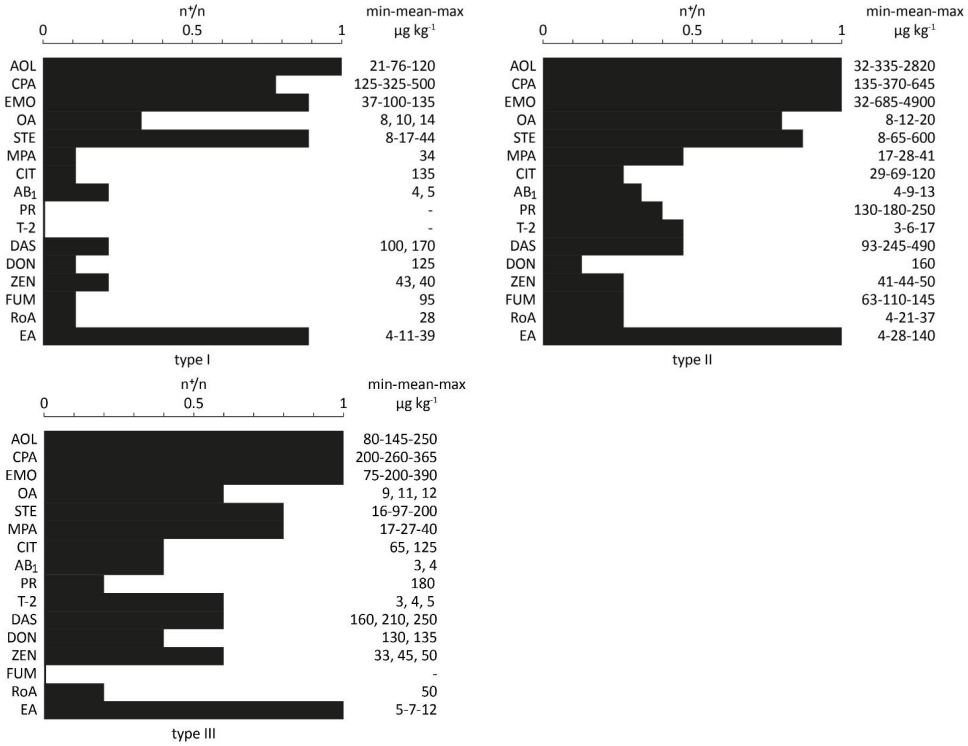

Fig. 1. Incidence and amounts of the mycotoxins before the first mowing in the samples of grasses (type I), clover-grass mixtures (type II), and alfalfa-timothy mixtures (type III). n = the total number of analyzed samples, n^+ = the number of samples where a mycotoxin is detected

The peculiarities of mycotoxin contamination found for field material of I-III types were confirmed by the results of sample analyzes that consisted of grass plants as well as individual clover and alfalfa plants collected from the same fields (Fig. 2). Indeed, the clover plants samples were in whole contaminated with AOL, CPA, EMO, OA, MPA, and T-2, and very extensive occurrences of STE, PR, DAS, FUM, and EA were found. The grasses revealed lower detection frequency of all mycotoxins except AOL and EA and very slight incidence of fusariotoxins and absence of PR. At the same time, amounts of AOL, CPA and especially EMO in comparison with clovers were much lower. Similarly to clover, AOL, CPA, EMO and EA were found in all the analyzed alfalfa samples, and MPA was also detected (Table 2). The other mycotoxins were revealed in one or two cases.

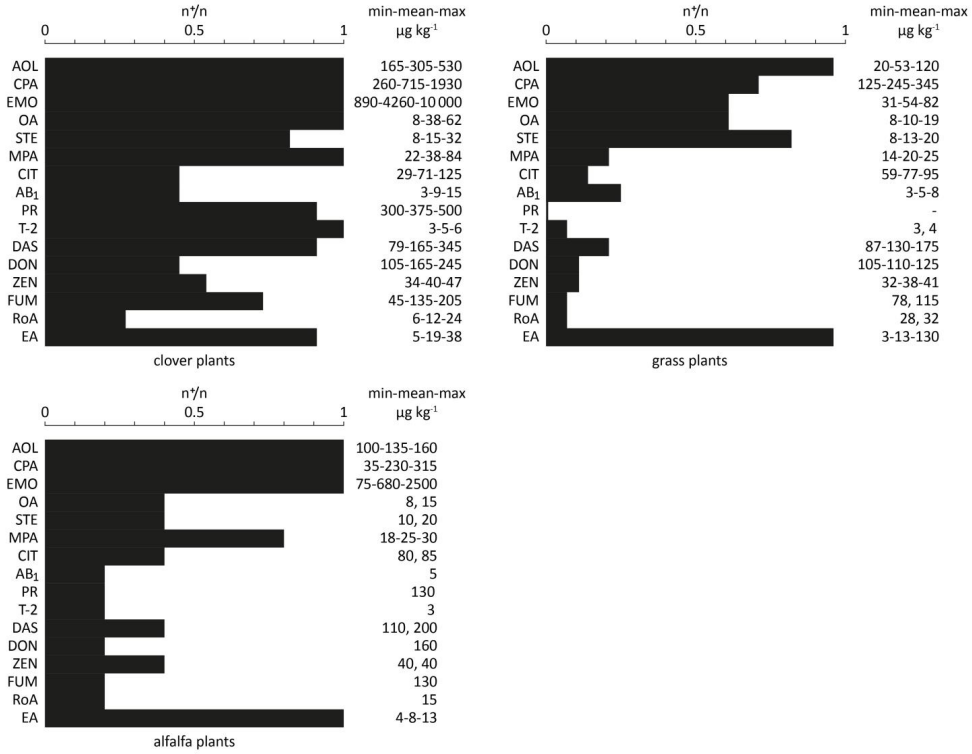

Fig. 2. Incidence and amount of the mycotoxins before the first mowing in the separate samples of grass, clover, and alfalfa plants. n = the total number of analyzed samples, n^+ = the number of samples where a mycotoxin is detected

The results of mycological analysis of forages before the second mowing are shown in Table 2. The values of total number of fungi in grasses and legume mixtures were quite different, and forage type II differed significantly from the others ($p < 0.05$). *Cladosporium* fungi prevailed in all types of second mowing herbage while incidence and quantity of other fungal taxa differed. In mixtures with clover, the intensity of contamination with *Acremonium*, *Alternaria,* and *Fusarium* was higher than in grasses. Fungi belonging to *Phoma*, *Aureobasidium,* and *Penicillium* genera were found in lower numbers. *Scopulariopsis* sp. was not detected in grasses, but the representatives of *Gliocladium* sp. appeared that had not been found before the first mowing.

Table 2. The mycobiota of different forage types before the second mowing

Taxon	type I (n = 3)		type II (n = 9)		type III (n = 3)	
	n^+	CFU g^{-1}	n^+	CFU g^{-1}	n^+	CFU g^{-1}
Acremonium spp.	3	8693	6	39422	3	5067
Alternaria spp.	2	1333	9	12133	2	1067
Aureobasidium sp.	1	267	2	178	1	133
Cladosporium spp.	3	39333	8	469644	3	6533
Fusarium spp.	2	1733	7	9778	3	533
Gliocladium spp.	2	1600	1	89	0	0
Mycelia sterilia	2	1600	5	5956	3	1200
Penicillium spp.	2	267	1	89	2	267
Phoma spp.	2	25467	2	14756	1	400
Scopulariopsis sp.	0	0	1	400	1	267
Other fungi	1	67	1	89	2	1200
Totally		80360		552533		16667

Type I = grasses; type II = clover-grass mixtures; and type III = alfalfa-timothy mixtures; n = total number of analyzed samples, n^+ = the number of samples where a taxon is found.

In terms of species composition of *Fusarium* fungi, type III differed from others forage types. In addition to *F. avenaceum*, *F. anguioides*, *F. sporotrichioides,* and *F. proliferatum,* revealed in all forage types, type III also contained *F. semitectum*, *F. culmorum*, *F. equiseti,* and *F. poae*.

The detection frequency and mycotoxin levels in forages before the second mowing are shown in Table 3. Both in the case of grasses and legume-based mixtures, the complex of four regularly occurring components, i.e., AOL, CPA, EMO, and EA was supplemented with FUM, MPA, and OA.

In grasses, only a single sample was contaminated with CIT and PR, while STE, RoA, AB_1 and four fusariotoxins (T-2, DON, DAS, and ZEN) were absent. In legume mixtures, concomitant toxins were found at lower frequencies or were completely absent (DAS and ZEN in clover mixtures and T-2, DAS, ZEN in alfalfa with timothy).

Table 3. Mycotoxins in different forage types before the second mowing

Mycotoxin	type I (n = 3)		type II (n = 9)		type III (n = 3)	
	n^+	min–mean–max, μg kg^{-1}	n^+	min–mean–max, μg kg^{-1}	n^+	min–mean–max, μg kg^{-1}
AOL	3	63, 144, 310	9	62–235–415	3	63,105,340
CPA	3	400, 1000, 1350	9	630–1050–1550	3	780, 1230, 1480
EMO	3	130, 1000, 2000	9	67–1270–2500	3	125, 200, 370
OA	3	8, 10, 17	8	10–17–26	1	20
STE	0	–	3	30, 32, 40	2	36, 46
MPA	2	17, 25	7	16–24–35	3	18, 22, 24
CIT	1	45	4	42–65–100	3	66, 84, 85
AB_1	0	–	3	2, 3, 4	2	3, 4
PR	1	250	4	250–310–385	1	165
T-2	0	–	3	4, 8, 36	0	–
DAS	0	–	0	–	0	–
DON	0	–	1	100	1	105
ZEN	0	–	0	–	0	–
FUM	3	75, 80, 165	8	68–105–160	2	85, 130
RoA	0	–	0	–	0	–
EA	3	6, 40, 60	9	14–47–130	3	3, 30, 60

Type I = grasses; type II = clover-grass mixtures; and type III = alfalfa-timothy mixtures; n = total number of analyzed samples, n^+ = the number of samples where a mycotoxin is detected

Discussion

Micromycetes belonging to six genera (*Alternaria*, *Aureobasidium*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Phoma*) were identified in all types of plant samples. *Cladosporium* fungi prevailed in all types of first and second mowing herbages, and about 50% of the identified CFU belonged to fungi of this genus. The presence of the other fungi (genera *Acremonium*, *Aspergillus*, *Gliocladium*, *Scopulariopsis* as well as *T. viride* and members of Mucoraceae family) varied in connection with the type of forage and time of mowing.

The numbers of micromycetes in grasses and clover-grass mixtures after their two-month (June-July) regrowth proved to be considerably higher than before ($p < 0.1$). The highest increase of incidences was noted for fungi of genera *Cladosporium* (especially in clover-grass mixtures), *Phoma*, *Fusarium*, and *Acremonium*. The tendency towards the increase in the number of fungi in the middle of vegetation period is rather explicable and may be the consequence of a manifold increase in their spore formation under favourable field conditions.

Nevertheless, in an alfalfa-timothy mixture (type III), the fungal contamination decreased, i.e., there was partial restoration over the same period. Especially the incidence of *Phoma* fungi dramatically reduced ($p < 0.1$). The only exclusion was the occurrence of *Acremonium* fungi in mycobiota, which were missing before the first mowing for the year. The different characters of mycobiota formation on various types of forage may be associated with peculiarity of microbial and host plant interactions, the mechanism of which has been poorly studied so far (Xu et al. 2007, Palumbo et al. 2008, Solomon 2011).

The changes of content and number of microorganisms in infested plants during the growing season, including toxigenic fungi, certainly has an impact on the range and amount of accumulated mycotoxins.

Indeed, the complexes of mycotoxins including up to 14–16 components and the combined character of plant contamination quite corresponds to the taxonomic variety of mycobiota. Currently, fusariotoxins, aflatoxins, and EA qualify as so-called field-derived mycotoxins, i.e., mycotoxins produced by fungi that colonizes the growing plants (Driehuis 2013). Our results showed that this group is much wider and includes all the analyzed mycotoxins, detected with different frequencies and quantities.

AOL, CPA, EMO, and EA formed the group of regular occurring mycotoxins. Invariably high incidence of them remains during the study period in all forage types. The earlier experiments revealed the same significant occurrence and levels of AOL, CPA and EMO in dry and fresh grasses (Kononenko and Burkin 2014a,b, Burkin and Kononenko, 2015). These metabolites are known for their toxicity to vertebrates and other animal groups. AOL found to possess genotoxicity, as well as mutagenic and teratogenic effects (EFSA 2011), EMO has a diarrheic effect (Wells et al. 1975), CPA leads to hepatic degeneration and necrosis, myocardial damage and exerts a neurotoxic effect by influencing the calcium metabolism and cellular conductivity processes (CAST 1989).

The contribution of fusariotoxins in the contamination appeared to be less significant. The majority of found *Fusarium* species are capable of producing fusariotoxins, albeit at different levels (Marasas et al. 1984, Leslie and Summerell 2006). Low ZEN content (< 50 μg kg^{-1}) was quite consistent with the data of Czech researchers who found it in freshly-mown grasses (ryegrass, festulolium, fescue, meadow-grass and their mixtures) in quantities from 5 to 48 μg kg^{-1} (Skladanka et al. 2013). Contamination of legumes and grasses with T-2 remained at equal background level (3–6 μg kg^{-1}), growth up to 36 μg kg^{-1} was found only in one case. Thus, the close toxin concentrations of 24–30 μg kg^{-1} were found in the fresh-cut material of selected forage grasses (Skladanka et al. 2013). We also detected low amounts of T-2 in haylage samples from 30 commercial feed batches from the livestock farms located in the central regions of the European part of Russia (Kononenko and Burkin 2014b). On the other hand, herbages were sometimes found to accumulate substantial amounts of T-2. For example, contamination of field-dried hay by T-2 reached as high as the values ranging between 500–700 μg kg^{-1} (Kononenko and Burkin 2014a).

Accumulation of DON and FUM was lower than that described for cereals grains though the quantities of DAS (which is usually not found in cereals) in grass plants reached 490 μg kg^{-1}. It must be noted that species, such as *F. avenaceum*, *F. anguioides,* and *F. tricinctum*, which are not the producers of T-2, DAS, DON, ZEN, and FUM, prevailed in grasses before the first mowing.

The experiments showed that before the first mowing of plant material, grasses and legumes exhibited specific features in the mycotoxin occurrence. Whole of the clover plants were contaminated with AOL, CPA, EMO, OA, MPA, T-2, and also very extensive presence of STE, PR, DAS, FUM, EA was found. The grasses revealed a lower frequency of all mycotoxins, except AOL and EA, as well as very slight incidence of fusariotoxins. Increased contamination of clover-based herbages of T-2, DAS, and FUM quite correspond to the infection caused by *F. sporotrichioides* and *F. proliferatum*. *Fusarium* species occurrence in clover mixtures before the second mowing led to T-2 detection, albeit infrequently and in small quantities, and extensive FUM contamination was revealed.

In few cases, accumulation level of mycotoxins did not coincide with the intensity of colonization with fungi. For example, amounts AOL in the clover-grass mixtures (type II) before the first mowing (up to 2820 μg kg^{-1}) were not in accordance with the lower intensity of their colonization with *Alternaria* fungi. It may be probably explained by the presence of high toxin producing fungal species and strains. Moreover, a part of isolated fungi (from 1 to 15%) were not identified due to a small set of morphological characteristics, homoplasy, and total absence of sporulation (Mycelia sterilia group). These fungi include *Acremonium, Alternaria, Arthrobotrys, Colletotrichum*, *Fusarium, Phyllosticta,* etc. (Lacap et al. 2003, Gao et al. 2010), and this may also have an impact on the potential contamination of plants. Macrocyclic trichothecene RoA was found in equally low contents in all forage types only before the first mowing.

EA with 100% incidence was found in quantities significantly lower than the levels that are possible in case of plants infected with *Claviceps* spp. It is known that other fungi can also be sources of this mycotoxin in grasses, including *Penicillium* and *Aspergillus* (Boichenko et al. 2001, Gerhards et al. 2014, Robinson and Panaccione 2015). EA is also produced by a group of endophytic fungi belonging to *Neotyphodium* and *Epichloe* genera (CAST 2003).

The incidence of STE in forages before the first mowing was high and the range of amounts were equal to 8-44 μg kg^{-1}. Detection of significant levels of STE in type II (600 μg kg^{-1}) and type III (200 μg kg^{-1}) forages are possibly linked to accompanying plants or weeds since such high concentrations were not found in separately collected samples of the legume plants.

Before the second mowing, there was the marked enhancement of the accumulation of CPA and EMO. There was increase in the intensity of contamination of MPA while the contribution of STE decreased. The changes of fusariotoxins composition were significant. There was a sharp increase in type II forage contamination with FUM while T-2 and DON detection frequency changed only slightly, but DAS and ZEN were not found at all. In forage type III (alfalfa and timothy), in spite of low fungal CFU in comparison with others forage types, the contamination with mycotoxins generally corresponded to that revealed before the first mowing, with the same four constant components, i.e., AOL, CPA, EMO, and EA. Another character of FUM distribution in the plants before the second mowing may be explained by increased spread of active producing species *F. proliferatum.*

Hence, the first and second mowing for grasses and legume plants resulted in multicomponent contamination with fungi and mycotoxins. In order to produce animal feed with high quality and safety standards, forage crops should be additionally evaluated by combining results of fungal and mycotoxin contamination analyzes. A better understanding of the environmental and cropping factors and the interaction between the representatives of plant mycobiota could contribute towards reducing the potential risk of the contaminated feed to the animal health.

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