Post-harvest Fungal Spoilage of Maize Silage

Species, growth conditions and mycotoxin detection

Storm, Ida Marie Lindhardt Drejer; Thrane, Ulf; Smedsgaard, Jørn

Publication date:
2009

Center for Microbial Biotechnology
Department of Systems Biology

DTU

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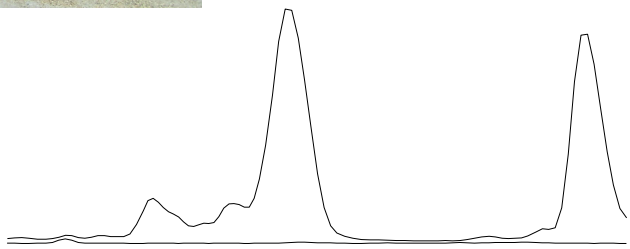
Ida ML Drejer Storm

PhD thesis

August 2009



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Preface

Filamentous fungi are ubiquitously present in our surroundings and they have both beneficial and harmful effects various places in nature. Spoilage of food and feed by filamentous fungi and subsequent contamination with mycotoxins is of great concern around the world. Fungal spoilage of feed may have dual effects, as some mycotoxins are transmitted to animal products for human consumption and may thus affect both animals and humans.

The joint project "Mycotoxin carry-over from maize silage via cattle into dairy products", supported by the Danish Directorate for Food, Fisheries and Agri Business, was initiated in 2005. The purpose was to ascertain whether mycotoxins in maize silage are causing illness and ill-thrift in dairy cattle and whether mycotoxins in the feed can be transferred to blood and milk. The participants of the project are the Technical University of Denmark, Aarhus University, Danish Agricultural Advisory Service and the Danish Plant Directorate.

This PhD thesis contributes to the project by exploring aspects of post-harvest fungal spoilage and mycotoxin contamination of maize silage under Danish conditions. The microbiological work was conducted at Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark and the majority of the chemical work was conducted at the Department of Food Chemistry, National Food Institute, Technical University of Denmark. A close co-operation with the other partners of the joint project was maintained throughout the PhD work, resulting in several joint studies. As a result of the general interest in the topic in agricultural circles three popular scientific articles in Danish have been published. They are included in Appendix D. The project has also involved many field trips.

This PhD study is financed by Danish Cattle Federation, the Research School FOOD and the Technical University of Denmark.

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Ida Marie Lindhardt Drejer Storm

Kgs. Lyngby, August 2009

Acknowledgements

My supervisors Ulf Thrane and Jørn Smedsgaard have been of invaluable help during my PhD study. With trust in my abilities they have guided me through without taking control. Besides Ulf's microbial knowledge I have benefitted greatly from his patient and pleasant manner. Jørn's analytical mind combined with a vast interdisciplinary overview has helped me with focusing on the important issues as well as discovering new interesting perspectives.

Many thanks are also directed to my external supervisor, Rudolf Thøgersen, at Danish Cattle Federation for connecting me to the real world of maize silage production and use through his knowledge and contacts.

The co-operation with Niels B. Kristensen and Birgitte M.L. Raun at Department of Animal Health and Bioscience, Aarhus University has also been very rewarding. The monitoring-study could not have been performed without their participance. Thank you also to Ole Green at Department of Agricultural Engineering, Aarhus University for letting me test his wireless silage sensors.

Huge thanks goes to my fellow PhD student Rie R. Rasmussen at the National Food Institute. Her work with the development of a multi-mycotoxin method has been very laborious. I look forward to continuing our co-operation.

At Center for Microbial Biotechnology I have benefitted greatly from the support and chemical knowledge of Kristian F. Nielsen. Jens C. Frisvad and Birgitte Andersen have been very helpful with identification of the many fungal isolates. The technical help by Jesper M. Mogensen and Jesper Mogensen with conducting the many microbial enumerations is also highly appreciated. The technical staff at CMB: Anni, Hanne, Kir and Lisette are also thanked for their many services, especially Lisette for supplying me with very large amounts of Petri dishes.

My previously fellow PhD student and office mate at CMB, Jens L. Sørensen, has also been a great support with both practical and theoretical issues. He is also thanked for going through the thesis with critical eyes.

In my family my gratitude goes particularly to my parents and grand-parents for their support in these last busy years and to my aunt Charlotte Lindhardt for reading and commenting the thesis.

Finally, thanks to my dear husband Adam Storm for the sacrifices he has made for me, especially during the last couple of months. And for being so ambitiously insisting that we can fulfil both scientific and personal dreams. Together we achieve wonderful things.

Dansk sammendrag

Siden 1990 er produktionen af majsensilage til fodring af kvæg steget med over 700% i Danmark. Desværre ses vækst af skimmelsvampe ofte i ensilage, og adskillige mykotoksiner er detekteret i ensilageprøver. Det er potentielt skadeligt for både kvæg, landmænd og forbrugere af mælk og oksekød.

I den første del af dette årti blev det diskuteret hvorvidt nogle uopklarede tilfælde af mistrivsel, sygdom og død i danske malkekvægs-besætninger kunne tilskrives mykotoksiner i ensilage. De generelt landbrugsfaglige og videnskabelige diskussioner gjorde det klart, at der manglede viden om dette emne under danske forhold. Formålet med dette studie er at afdække vækst og udbredelse af skimmelsvampe i danske majsensilagestakke og at udforske produktionen af post-harvest mykotoksiner i majsensilage.

De aktuelle skimmelsvampearter i dansk majsensilage og deres frekvens *in situ* blev estimeret ved monitorering af 20 majsensilagestakke gennem en hel ensilagesæson. Dyrkbare enheder af skimmelsvampe var tilstede i alle 20 stakke gennem hele studiet. De mest almindelige arter var *Penicillium roqueforti* og *Penicillium paneum*, Zygomyceter og *Aspergillus fumigatus. Byssochlamys nivea*/*Paecilomyces niveus*, *Monascus ruber* og *Geotrichum candidum* forekom mindre hyppigt. Antallet af gær og mælkesyrebakterier blev også bestemt, men disse mikroorganismer blev ikke nærmere identificeret.

Heterogeniteten af mikrobielle parametre og foderværdi i majsensilage blev undersøgt i et separat studie. Formålet var at evaluere den anvendte samplingsprocedure og vurdere den generelle usikkerhed ved bestemmelse af foderværdien og af antallet af kolonidannende enheder af skimmelsvampe, gær og mælkesyrebakterier i ensilagestakke af fuld størrelse. Antallet af kolonidannende enheder af skimmelsvamp og gær varierede meget både imellem og indenfor stakke. Dette studie angiver at der skal tages mere end 11 boreprøve i fuld dybde af en ensilagestak for at bestemme gennemsnitskoncentrationen så den med 95% sikkerhed ikke afviger mere end ±1 logCFU fra den reelle værdi. Mælkesyrebakterierne og de fysisk/kemiske mål for foderværdien var langt mere homogent fordelt end svampene både indenfor og mellem stakke.

Antallet at dyrkbare enheder af skimmelsvampe i prøverne fra de 20 stakke i monitoreringsforsøget blev testet for korrelation med de fysiske og kemiske egenskaber i prøverne og med antallet af gær og mælkesyrebakterier i dem. De gennemsnitlige værdier for alle de mikrobielle parametre varierede signifikant mellem de 5 samplingstidspunkter i løbet af sæsonen. Antallet af kolonidannende enheder af svampe (gær og skimmel) var højest i 5-7 måneder gammel ensilage og signifikant lavere i 11 måneder gammel ensilage. Hyppigheden af hot-spots med synlig vækst af skimmelsvamp fulgte den samme tendens. Der blev ikke fundet nogle bemærkelsesværdige korrelationer mellem antallet af dyrkbare enheder af skimmelsvamp og parametrene: antal af mælkesyrebakterier, antal af gær, tørstofindholdet, pH, temperatur 15 cm bag snitfladen, og koncentrationerne af ethanol, propanol, 2-butanol, propanal, ethylacetat, propylacetat, propylen glycol, D-glucose, L-lactat, ammoniak, acetat, propionat og butyrat.

Gassammensætningen inden i ensilagestakke har stor betydning for væksten af skimmelsvampe i ensilage. Forholdet mellem gasser forskydes let, for eksempel i forbindelse med huller i ensilagens indpakning. To forskellige metoder til *in situ* måling af A) O_2 og CO_2 og B) temperatur og O_2 blev

testet. Metode A anvendte en håndholdt gasdetektor med elektrokemisk O_2 sensor og infrarød CO_2 sensor. Metode B var en prototype af en trådløs temperatur og O_2 sensor udviklet til ensilage. Metode A var i stand til at lave omtrentlige målinger af O_2 og CO_2 *in situ*, men yderligere test af metoden er nødvendigt for at bestemme dens nøjagtighed. Sensorerne i metode B var i stand til at monitorere og transmittere ensilagetemperaturen kontinuert gennem 53 dage og sensorerne var funktionelle i mindst 102 dage. O_2 sensoren i den anvendte prototype kunne ikke modstå det syreholdige miljø i ensilagen og holdt hurtigt op med at fungere.

Endelig blev der udviklet og valideret en multi-mykotoksin metode til bestemmelse af 27 svampemetabolitter i majsensilage ved hjælp af HPLC-MS/MS. Metoden inkluderer sekundære metabolitter fra alle de mest almindelige post-harvest svampe i majsensilage, undtagen zygomyceterne, samt adskillige pre-harvest mykotoksiner med relevans for majs. Atten analytter blev valideret kvantitativt og 9 kvalitativt. Metoden blev derefter anvendt til analyse af 4 svampe hot-spots fra majsensilage. Svampene *Penicillium roqueforti*, *Penicillium paneum*, *Byssochlamys nivea* og *Monascus ruber* blev isoleret fra prøverne og post-harvest metabolitterne andrastin A, citreoisocoumarin, marcfortine A and B, mycophenolic acid and roquefortine A and C blev identificeret sammen med pre-harvest mykotoksinerne enniatin B, nivalenol, zearalenone og deoxynivalenol. Den højeste målte koncentration var 34 ± 18 $mg \cdot kg^{-1}$ af roquefortine C.

På basis af disse resultater er det ikke muligt at fastslå hvorvidt de observerede tilfælde af sygdom og mistrivsel i malkekvægsbesætninger kan være forårsaget af post-harvest mykotoksiner i majsensilage. Yderligere analyser af ensilageprøver og mere information om de toksikologiske effekter af mykotoksiner på kvæg er nødvendige. Potentialet for vækst af skimmelsvampe er generelt til stede i alle majsensilagestakke. Dette understreger vigtigheden af grundighed og gode procedurer i forbindelse med produktion og anvendelse af ensilage. Da størstedelen af de observerede skimmelsvampe i stakkene fandtes i de ydre lag, er det muligt for landmanden at kassere dem før fodring. Dette vil mindske mængden af kontamineret ensilage der når kvæget betydeligt. Resultaterne indikerer også at risikoen for vækst af skimmelsvamp i velforgærede stakke af majsensilage kan reduceres ved at holde stakkene forseglede i mere end 7 måneder, før de åbnes.

Summary

From 1990 to 2008 the production of maize silage for cattle feed in Denmark has increased by more than 700 percent. Unfortunately, growth of filamentous fungi is often seen in silage and mycotoxins have been detected in silages. This is potentially harmful to livestock, farmers and consumers of dairy and meat products.

In the beginning of this decade concern was raised whether unexplained cases of ill-thrift, disease and death in Danish dairy herds were caused by mycotoxins in silage. Public and scientific discussions about the problem revealed that more information on this issue under Danish conditions was needed. This study aims at exploring the extend and growth of filamentous fungi in Danish maize silages and the post-harvest contamination of maize silage with mycotoxins.

The species of filamentous fungi occurring in Danish maize silages and their frequency *in situ* was examined by monitoring 20 maize silage stacks over a whole season. Viable propagules of filamentous fungi were present in all silage stacks at all times during the study. The most frequent species were *Penicillium roqueforti* and *Penicillium paneum*, Zygomycetes, and *Aspergillus fumigatus. Byssochlamys nivea*/*Paecilomyces niveus*, *Monascus ruber* and *Geotrichum candidum* occurred less frequently. Yeasts and lactic acid bacteria were also enumerated but not identified.

The heterogeneity of microbial and feed value parameters in maize silage was examined in a separate study in order to evaluate sampling procedures. The numbers of colony forming units (CFU) of filamentous fungi and yeasts varied substantially between and inside maize silage stacks. The study suggests that more than 11 samples are needed from one stack to determine an average concentration of filamentous fungi which is 95% certain to be within ± 1 logCFU. Lactic acid bacteria and feed value parameters were much more homogenously dispersed than the fungi both inside and between stacks.

Numbers of viable propagules of filamentous fungi detected during the monitoring of 20 maize silage stacks were tested for correlation with physical and chemical properties of the silage samples, as well as the CFUs of yeast and lactic acid bacteria. The average counts of all microbial parameters were shown to vary significantly over a storage season. The amounts of colony forming units of fungi were highest in 5-7 month old silage and significantly lower in 11 month old silage. The occurrence of hot-spots with visible fungal growth showed the same tendency. There were no noteworthy correlations between numbers of cultivable units of filamentous fungi and any of the parameters: counts of lactic acid bacteria, counts of yeasts, dry matter content, pH, temperature 15 cm behind bunker face and concentrations of ethanol, propanol, 2-butanol, propanal, ethyl acetate, propyl acetate, propylene glycol, D-glucose, L-lactate, ammonia, acetate, propionate and butyrate.

A factor which is known to have an important effect on the growth of filamentous fungi in silage is the atmospheric composition inside the silage. This composition is easily disturbed by for example holes in the silage cover. Ingress of air leads to increased microbial activity which releases heat. Two different methods for *in situ* detection of A) O_2 and CO_2 and B) temperatur and O_2 in maize silage stacks were tested. Method A employed a hand-held gas detector fitted with an electrochemical oxygen sensor and an infrared carbon dioxide sensor. Method B was a proto-type wireless

temperature and oxygen sensor for silage. Method A was capable of making approximate measurements of O_2 and CO_2 *in situ*, but further testing of the procedure is necessary to ascertain the accuracy of the measurements. Method B sensors were capable of monitoring and transmitting silage temperature continuously for 53 days and the sensors were functional for at least 102 days. The oxygen sensor in this prototype could not withstand the acidic environment of the silage.

Finally, a multi-mycotoxin method for the detection of 27 fungal metabolites in maize silage by liquid chromatography and tandem mass spectrometry was developed and validated. The method covers secondary metabolites from all the most common post-harvest fungal contaminants of maize silage, except the Zygomycetes, as well as several pre-harvest mycotoxins. Eighteen of the analytes were validated quantitatively and 9 qualitatively. It was subsequently applied to four fungal hot-spots from maize silage. The fungi *Penicillium roqueforti*, *Penicillium paneum*, *Byssochlamys nivea* and *Monascus ruber* were isolated from the samples and the post-harvest fungal metabolites andrastin A, citreoisocoumarin, marcfortine A and B, mycophenolic acid and roquefortine A and C were identified together with the pre-harvest mycotoxins enniatin B, nivalenol, zearalenone, and deoxynivalenol. The highest concentration detected was 34,000 ± 18,000 $\mu g \cdot kg^{-1}$ of roquefortine C.

On the basis of the present results it is not possible to ascertain whether post-harvest mycotoxins in maize silage can be the cause of the observed incidents of illness and ill-thrift in dairy cattle. Mycotoxin determination in many more samples and more information on the toxicological effects of post-harvest mycotoxins in cattle is needed. The potential for fungal growth is generally present in all silage stacks, emphasising the importance of proper silage management. As the majority of the filamentous fungi observed *in situ* were present in the outer layers of silage stacks it is possible to discard them prior to feeding. Thereby, the amount of contaminated silage in the feed is reduced. The results also suggest that the risk of fungal spoilage of well fermented maize silage can be limited by keeping stacks well sealed for more than seven months before opening.

List of original papers

I. Storm, I.M.L.D., Sørensen, J.L., Rasmussen, R.R., Nielsen, K.F., and Thrane, U. (2008) Mycotoxins in silage. *Stewart Postharvest Review* **6**(4).

II. Storm, I.M.L.D., Kristensen, N.B., Raun, B.M.L., Smedsgaard, J., and Thrane, U. Dynamics in the microbiology of maize silage during whole-season storage. *Submitted to Microbial Ecology*.

III. Storm, I.M.L.D., Thøgersen, R., Smedsgaard, J., and Thrane, U. Intra-stack heterogeneity of microbial and feed value parameters of maize silage. *Submitted to Animal Feed Science and Technology.*

IV. Green, O., Nadimi, E.S., Blanes-Vidal, V., Jørgensen, R.N., Storm, I.M.L.D., and Sørensen, C.G. (2009). Monitoring and modeling temperature variations inside silage stacks using novel wireless sensor networks. *Accepted by Computers and Electronics in Agriculture.*

List of popular scientific publications in Danish

D-I Ulf Thrane, Ida ML Drejer Storm and Jens L Sørensen (2006) Svampe og deres toksiner i majsensilage. KvægInfo, no. 1633.

D-II Ida ML Drejer Storm and Rudolf Thøgersen (2008) Forekomst af skimmelsvamp i majsensilage. Ny Kvægforskning **6**(6).

D-III Jens L Sørensen, Ida ML Drejer Storm, Birgitte Andersen and Ulf Thrane (2009) Marksvampe og deres mykotoksiner i majs. Ny KvægForskning **7**(4).

Abbreviations

CFU	Colony forming units
DG18	Dichloran glycerol 18% agar medium
DM	Dry matter
DON	Deoxynivalenol
ESI	Electro Spray Ionisation
ESI-	Negative Electro Spray Ionisation
ESI+	Positive Electro Spray Ionisation
FK	Freerslev Kotel I/S
GG	Gjorslev Gods
HT-2	HT-2 toxin
LAB	Lactic acid bacteria
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of Detection
MeCN	Acetonitrile
MRM	Multiple Reaction Monitoring
MRS	Mann, Rogosa, Sharp medium
MYGP	Malt Yeast Glucose Peptone medium
NDF	Neutral detergent fibre
NIR	Near infrared spectroscopy
OMD	Organic matter digestibility
PCR	Polymerase Chain Reaction
PSA	Primary, Secondary Amine
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
SPE	Solid phase extraction
T-2	T-2 toxin
V8	V8-juice medium
WSC	Water soluble carbohydrates

Contents

1 Introduction

Maize silage is a widely used feed product for cattle in dairy and meat production. Silage is to some extent displacing the use of hay (Wilkinson and Toivonen, 2003) as the process of ensiling is much less weather dependent (Wilkins et al., 1999). In Western Europe in the year 2000 the productions of hay, grass silage and maize silage were almost equal at approximately 50 million metric tonnes dry matter (DM) of each (Wilkinson and Toivonen, 2003). For North America the corresponding amounts were approximately 180, 2 and 35 million tonnes of dry matter, respectively (Wilkinson and Toivonen, 2003). In Denmark the production of maize for silage has increased by more than 700% from 1990 to 2008 (Figure 1.1). With maize silage as the primary ingredient in a total mixed ration for dairy cattle and a dry matter intake per cow of 20 kg·day^{-1} a normal dairy cow can easily eat 20 kg·day^{-1} of maize silage and in extreme cases as much as 40 kg·day^{-1} (Rudolf Thøgersen, Danish Cattle Federation, Personal communication). For a dairy farm with 100-200 cows, this amounts to several tons a day.

It is thus clear that maize silage is a very important feed product. Fungal spoilage of maize silage may therefore have severe implications for both livestock, farmers and potentially for consumers of dairy and meat products.

1.1 Production and use of maize silage

Around the world different types of maize silage are produced, depending on maize types, ripeness at harvest and the use of separate fractions of the maize plants (Roth et al., 1995; Wilkinson and Toivonen, 2003). In Denmark the most common type of maize silage is produced from whole plants, which are harvested at a DM content of 30-35% (Nielsen et al., 2003). The maize is planted in May and reach the desired DM level in September or October in the Danish climate. This typical type of

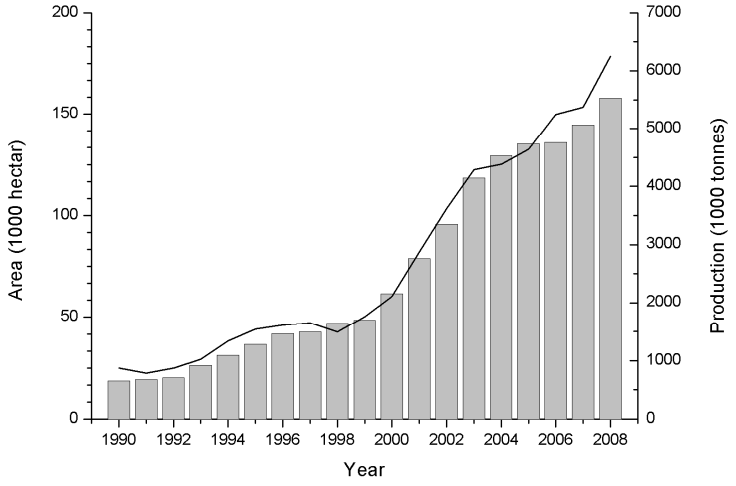

Figure 1.1: Cultivated area (bars) and production (−) of maize for silage in Denmark from 1990 to 2008 (Statistics Denmark, 2009).

Figure 1.2: Production and use of maize silage, from harvesting of whole-crop maize (upper left) and ensiling in a bunker silo (lower left), to a field stack in use (upper right) and cows eating silage (lower right).

Danish whole crop maize silage will hence forth be referred to as maize silage.

The maize plants are harvested 15-20 cm above the ground and chopped finely using specialised agricultural machinery (Figure 1.2). The plant material subsequently need to be heavily compacted and sealed from atmospheric air. This can be done in silos, planar silos, stacks on the ground or plastic sleeves (Weinberg and Ashbell, 2003). The most common practices in Denmark are planar silos on concrete platforms with one to three concrete walls, or field stacks of variable dimensions, laid either on the bare ground or on concrete.

The loads of chopped maize are unloaded at the selected site and spread out in thin layers, preferably no more than 10 cm thick at a time (Nielsen et al., 2003). Each layer is then compacted by driving over it with heavy machinery. This removes atmospheric air which facilitates a rapid subsequent fermentation and reduces the risk of oxygen ingress after opening of the stack or silo for feed-out.

To further prevent oxygen ingress the silage is covered with plastic, usually polyethylene of 0.1 -0.2 mm in thickness (Weinberg and Ashbell, 2003). The Danish Agricultural Advisory Service recommends two or three layers of UV-stabilised polyethylene with a minimum thickness of 0.15 mm (Nielsen et al., 2003). Often a thin plastic film (0.05 mm) is used directly on the silage, where it clings to the surface, while the thicker sheets on top provide physical protection from weather, animals, man and machines. Often protective nets are also applied. These prevent physical damage to the plastic from e.g. birds and weigh down the plastic. Finally sandbags or discarded tyres are used to weigh down the plastic and nets, and keep a slight pressure on the upper layers of silage.

The ensiled maize should be left sealed for a minimum of 3-4 weeks before opening of the stacks and feed out (Nielsen et al., 2003). During this time the silage undergoes microbial and chemical changes (see chapter 1.2) and stabilises at a pH below 4 with O_2 concentrations of a few percent and

CO_2 concentrations initially as high as 70-90% (Weinberg and Ashbell, 1994). At this stage the silage is considered stabile and stacks can be opened for feed out.

Plastic and other covers are removed from a small part of the stack while remaining covers are kept in place with tyres, sandbags or bales of straw. There are different types of machinery for removing silage from the stacks. Commonly used are front-loading tractors but they have a tendency to disturb the surface of the stack and leave a rough surface more prone to ingress of air. Specialised scrapers or block cutters leave a more compact cutting surface of the silage stack (Nielsen et al., 2003; Weinberg and Ashbell, 2003).

After removal the silage is mixed with remaining feed components to a total mixed ration, which is fed to the cattle.

1.2 The process of ensiling

The conversion from freshly harvested maize to maize silage is the result of many naturally occurring enzymatic and microbiological processes. The biochemical feasibility and timing of these processes are of utmost importance for proper preservation of the silage.

Pre-harvest

Maize plants harvested at the correct stage are excellent crops for silage as they have a high DM content, adequate concentrations of water soluble carbohydrates (WSC) for lactic acid fermentation and have a low buffering capacity allowing rapid acidification (McDonald et al., 1991). The

Table 1.1: Averages, 10% quantiles and 90% quantiles of selected physical and chemical parameters for Danish clover grass (1st cut) and maize silage samples analysed in 2008 (Kjeldsen and Thøgersen, 2009), illustrating differences between the two feed products.

	Maize silage (n=3800/3719)[a]			Clover grass, 1st cut (n=1761)		
	Mean	10% quantile	90% quantile	Mean	10% quantile	90% quantile
Harvest date	06/10	23/09	23/10	23/05	14/05	01/06
Dry matter ($g \cdot kg^{-1}$)	335	293	377	410	302	526
Ash ($g \cdot kg\ DM^{-1}$)	33	27	40	86	70	104
Crude protein ($g \cdot kg\ DM^{-1}$)	78	69	88	150	121	181
Crude fiber ($g \cdot kg\ DM^{-1}$)	189	165	214	226	200	253
Starch ($g \cdot kg\ DM^{-1}$)[b]	326	271	380	-	-	-
WSC ($g \cdot kg\ DM^{-1}$)[bc]	-	-	-	120	46	191
NDF ($g \cdot kg\ DM^{-1}$)[c]	376	333	422	380	332	432
OMD[c]	0.773	0.749	0.793	0.797	0.759	0.828
pH	3.78	3.60	3.90	4.60	4.10	5.10
Lactic acid ($g \cdot kg\ DM^{-1}$)	49	38	61	52	11	88
Acetic acid ($g \cdot kg\ DM^{-1}$)	14	9.0	20	12	1	22
Ammonia-N ($g \cdot kg\ total\ N^{-1}$)	39	26	53	50	21	77

[a]Only 3719 samples analysed for pH, lactic acid, acetic acid and ammonia-N

[b]Starch is only determined for maize silage, water soluble carbohydrates are only determined for grass silage.

[c]WSC: Water soluble carbohydrates; NDF: Neutral detergent fiber; OMD: Organic matter digestibility

differences between grass and maize silage, as the two most common types, should also be noted (Table 1.1). Grass silage is often wilted in the field prior to ensiling and may have very variable DM contents (Table 1.1), and often grass is ensiled without chopping. The content of WSC is higher in grass, whereas starch content is higher in maize (McDonald et al., 1991)(Table 1.1). Even though the principles of the ensiling process are the same regardless of crop, there are differences, and conclusions drawn regarding one crop do not necessarily apply to the other.

Prior to harvest and ensiling the amount of lactic acid bacteria (LAB) on the plants is low. McDonald (1991) reviews the literature and indicates counts of colony forming units (CFU) of LAB in the range $1 \cdot 10^2$ to $1 \cdot 10^3$ CFU·g-^{1} for standing crops and about 10-fold higher in chopped crops. In a comparison of LAB numbers and species on grass and maize at different times of harvest (Ruser, 1989) average values of $2 \cdot 10^{-5}$ CFU·g^{-1} and $7 \cdot 10^{-4}$ CFU·g^{-1} were obtained for chopped maize and grass, respectively, indicating some differences between crops in the initial LAB population. Lin et al. (1992) finds LAB CFUs in the range 10^3 to 10^5 on standing maize and around 10^6 after chopping and LAB counts of 10^5-10^6 were recorded by Dellaglio and Torriani (1986) prior to ensiling of chopped maize. The chopping process is believed to raise the LAB CFU almost immediately by releasing nutrients from the plant material that may either act as substrate for multiplication or as protective agents against atmospheric oxygen (McDonald et al., 1991). Lower counts of LAB are also obtained on e.g. Rogosa agar than on less selective LAB media like Mann, Rogosa, Sharp agar (MRS)(Seale et al., 1990). The very low numbers of LAB detected on plants in older studies may therefore be due to both effect of the selected media and deterioration of LAB between harvesting of whole plants and laboratory analysis.

Other bacteria present on the plants prior to harvest are Enterobacteria, also known as coliform bacteria, and the spore forming *Clostridia* and *Bacillus* (McDonald et al., 1991; Seale et al., 1990).

The aerobic phase

After chopping of the maize plants, the natural enzymatic processes of the plant metabolism continue. In the presence of O_2 immediately after harvest and in the initial period of ensiling, glucose is metabolised, converting it to CO_2, water and energy, which is released primarily as heat (McDonald et al., 1991). Enzymatic activity of the plant cells has also been shown to degrade structural carbohydrates of grass to WSC during ensiling (McDonald et al., 1991). Enzyme activities vary with moisture content, temperature and stage of growth. Compared to grass, maize plants are mature, sometimes partly wilted, and the temperatures are on average 1.65^{o}C lower in September/October than in May/June (DMI, 2009), when grass is usually harvested. The sun also shines more in May and June than in autumn, where precipitation is highest. Therefore the effect of enzymatic activity in maize plants at harvest under Danish conditions is difficult to predict.

After compaction and sealing of the crop the aerobic phase is very short, primarily due to the enzymatic activity. According to Sprague (1974) cf. (McDonald et al., 1991) most of the oxygen initially present in a laboratory silo disappeared within 15-30 minutes. O_2 concentrations below 2% have been registered throughout months of silage storage while CO_2 concentrations are initially as high as 70-90% but often decrease over time (Forristal et al., 1999; Weinberg and Ashbell, 1994). According to Weinberg and Ashbell (1994) the concentration of CO_2 may vary considerably between silos of maize silage, probably due to differences in compaction and crop properties.

The anaerobic phase

The initiation of the anaerobic phase leads to major changes in the microbiology of the silage. The LAB and yeasts initiate fermentation and multiply rapidly, as illustrated in Figure 1.3. LAB generally peak at CFUs of app. 10^8-10^{10} within a few days (Dellaglio and Torriani, 1986; Lin et al., 1992; McDonald et al., 1991; McEniry et al., 2008a; Muck et al., 1992). The number of yeasts may vary substantially between silages. Muck et al. (1992) had maximum yeast counts ranging from 10^4 to 10^8 within the first 4 days for maize from different sources. Also the Enterobacteria multiply rapidly within the first few days and peak at concentrations of 10^7–10^9 CFU·g^{-1} (Byrne et al., 2002; Lindgren et al., 1985b; Muck et al., 1992) or 10^5-10^6 (McEniry et al., 2008a) in grass silage.

With the rapid proliferation of LAB, concentrations of lactic and acetic acid increase and pH decreases. In maize silage a pH of 4 or lower is obtained in 3-5 days after which it stabilises at this level (Dellaglio and Torriani, 1986; Driehuis et al., 1999; Lin et al., 1992; Middelhoven and Baalen, 1988; Muck et al., 1992). In grass silage the final pH is often a bit higher (Table 1.1) and the decrease more graduate (McEniry et al., 2008a) due to a higher buffer capacity of grass (McDonald et al., 1991; Nielsen et al., 2003).

The LAB can be divided in two groups: the homofermentative and the heterofermentative LAB on the basis of the metabolic pathways employed during fermentation. The homofermentative LAB convert hexose sugars to lactic acid employing the enzyme fructose bisphosphate aldolase. The heterofermentative convert hexose and pentose sugars to primarily lactic and acetic acid as well as CO_2 and ethanol (Carr et al., 2002; Holzer et al., 2003; McDonald et al., 1991). An intermediary

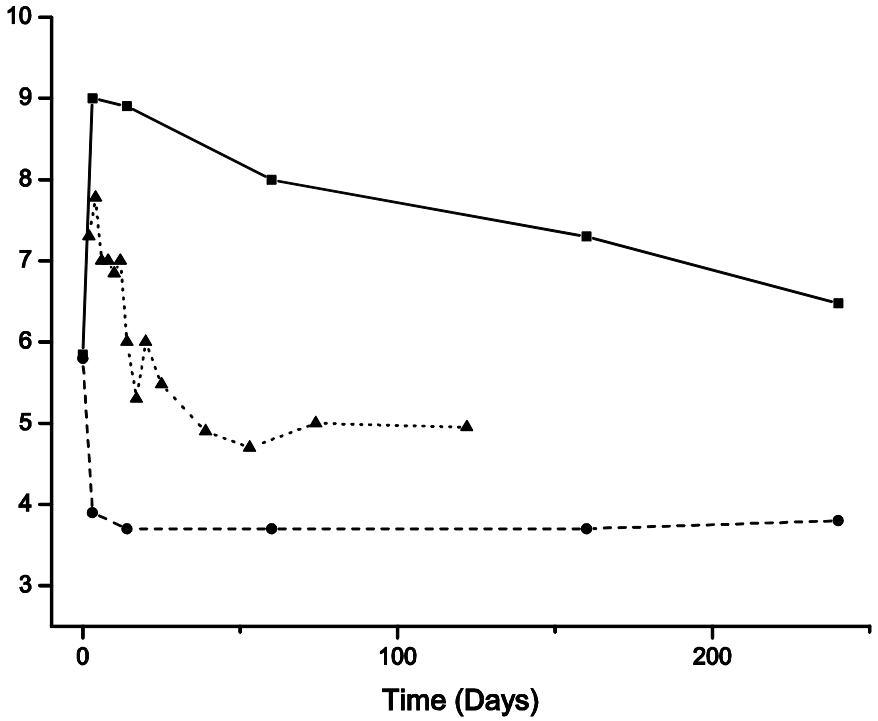

Figure 1.3: Illustration of the development in pH (●), number of yeasts (▲) and numbers of lactic acid bacteria (■) in maize silage from day 0-240 of the ensiling process. Modified from Middelhoven and van Balen (1988) and Dellaglio and Torreani (1986).

category termed facultative heterofermentative LAB is described by McDonald et al. (1991) and Holzer et al. (2003). This group primarily use the homofermentative pathway but can switch to heterofermentative metabolism under certain conditions. The lowering of pH leads to changes in the composition of the microbial population. The literature is not conclusive on these developments in the species composition as large variations occur between cultivars, regions and time of harvest (Lin et al., 1992; Ruser, 1989; Stevenson et al., 2006). Various studies indicate that the homofermentative LAB dominate early in the ensiling process while the heterofermentative constitute a larger proportion of the LAB at later stages, as indicated by (Holzer et al., 2003; Lindgren, 1990; McDonald et al., 1991).

It appears that LAB diversity is quite high prior to ensiling. Brusetti et al. (2006) and Lin et al (Lin et al., 1992) found large proportions of *Pediococcus pentosaceus*, and Leuconostocs were the dominant group found by Ruser (1989) on maize. Ruser (1989) detected more heterofermentative LAB on maize than on grass. Lin (Lin et al., 1992) also found *Enterococcus faecium* and *E. faecalis* in high proportions. Chopping of the crop changes the relative proportions of these species to each other, but not the occurrence(Lin et al., 1992). In the very early stages of ensiling LAB diversity is still large with findings of Leuconostocs, Streptococci, Pediococci, Lactobacilli and Enterococci (Dellaglio and Torriani, 1986; Holzer et al., 2003; Lin et al., 1992; Lindgren, 1990; Stevenson et al., 2006). As pH falls the acid tolerant Lactobacilli begin to dominate (Holzer et al., 2003; Lin et al., 1992; Rossi and Dellaglio, 2007; Stevenson et al., 2006). One commonly found species is the homofermentative *Lactobacillus plantarum* (McDonald et al., 1991). In some cases the ecological niche is taken over by heterofermentative LAB species e.g. *L. brevis* or *L. buchneri* over a time-span of weeks and months (Dellaglio and Torriani, 1986; Holzer et al., 2003; Lindgren, 1990). *L. buchneri* is capable of assimilating lactate under anaerobic conditions, turning primarily converting it to acetate and 1,2-propanediol (Elferink et al., 2001). On a longer time-scale the total number of LAB falls and after 2-3 months LAB numbers are 10-1000 times lower than the maximum count on day 3-5 (Dellaglio and Torriani, 1986; McEniry et al., 2008b; Muck et al., 1992).

Yeasts are very pH tolerant and some species can continue to grow at pH values as low as 3.5 (McDonald et al., 1991). Yeast encountered in maize silage can assimilate lactic and acetic acid and most of them can tolerate a medium with pH 4 containing both lactic and acetic acid (Middelhoven and Franzen, 1986). As for the LAB there is a shift in the yeast species encountered during the course of ensiling. The initial non-fermenting yeasts of the fresh crop are replaced by fermentative species and the relative proportions of these yeasts changes during storage (Middelhoven, 1998). However, silage is a hostile environment even for yeast and their numbers decrease slowly over the first couple of months (Middelhoven and Baalen, 1988). The low pH effectively inhibits the Enterobacteria and counts below 10 $CFU \cdot g^{-1}$ have been detected in maize and grass silages within 3 and 35 days, respectively (McEniry et al., 2008a; Muck et al., 1992).

During good ensiling the Clostridial counts do not increase in line with the previously mentioned organisms (Jonsson, 1991; Lindgren et al., 1988). Their growth is inhibited at a pH around 4 (McDonald et al., 1991). Therefore a low buffer capacity of the crop decreases the risk of Clostridial spores in the silage, so maize should be less prone to this bacterial contaminant than other crops. Less than 10^3 anaerobic spores per gram silage is considered a low concentration giving very little risk of spores in milk (Nielsen et al., 2003).

Little data is available on the developments in silage microbiology after the initial 3-4 months. Pahlow et al. (2003) states that this stable phase in theory can be of any length, as long as there is sufficient substrates for the LAB. Changes can however take place in the microbiota of silage during this phase. A monitoring of maize silage over 240 days reveals a continuous drop in LAB numbers after the day 3-5 peak (Dellaglio and Torriani, 1986). A continuous decline in yeast numbers from day 4 to 122 was also observed (Middelhoven and Baalen, 1988).

1.3 Spoilage of maize silage

In the previous section the ideal development of well produced and managed silage is described. Unfortunately silages are prone to spoilage by different microorganisms. This may lead to degradation of the feed which constitutes a loss of DM in itself and can reduce the palatability of the feed to cows. Spoiled silage can also contain bacteria and filamentous fungi which may endanger the health of livestock, farmers and possibly consumers of meat and dairy products.

Aerobic deterioration

In a well ensiled stack or silo of maize silage pH is below 4, O_2 is below 1-2% and CO_2 above 20%. This effectively stops fungi from multiplying and degrading the silage. But small flaws in the sealing of the stack or improper management can lead to aerobic deterioration of the silage.

With the availability of O_2, yeasts and acetic acid bacteria begin to degrade lactic and acetic acid as well as remaining WSC, converting it primarily to CO2, water and heat (McDonald et al., 1991). Acetic acid bacteria have been shown to be able to initiate aerobic deterioration of silage but they are often present together with yeasts and are outnumbered by these (Spoelstra et al., 1988). As the acids disappear, pH rises and opens up for the growth of Clostridia, Bacilli and Enterobacteria as well as other yeasts and filamentous fungi. This may eventually lead to complete microbial deterioration of the affected silage.

To illustrate the typical onset of aerobic deterioration of maize silage upon exposure to atmospheric air a theoretical graph of microbial and chemical parameters during aerobic deterioration is compiled on the basis of experimental results from the literature (Figure 1.4). The concentrations of organic acids, WSC and ethanol gradually decrease as the number of yeasts increases, and the increased microbial activity results in higher temperature. When concentrations of organic acids are sufficiently low pH begins to rise giving bacterial contaminants better conditions for further degradation.

In an experiment by Driehuis et al. (1999) an increase in O_2 concentrations in 98 days old maize silage from <0.5% to 2.5% led to increased numbers of yeasts within 4 days. As shown by Weinberg and Ashbell (1994) and the diffusion model by Pitt and Muck (1993) oxygen can diffuse more than one meter into silage stacks from the bunker face. The initial stages of aerobic deterioration can therefore take place days before the silage is actually exposed.

Some properties increasing the aerobic stability of silages have been determined. The speed of aerobic deterioration is believed to be dependent on the number of yeasts present before aerobic exposure, more yeasts leading to faster spoilage (McDonald et al., 1991; Pitt et al., 1991). Yeast numbers alone can not explain variations in aerobic stability. There are reports of stable silages with high yeast counts indicating that the species of yeast are important (McDonald et al., 1991).

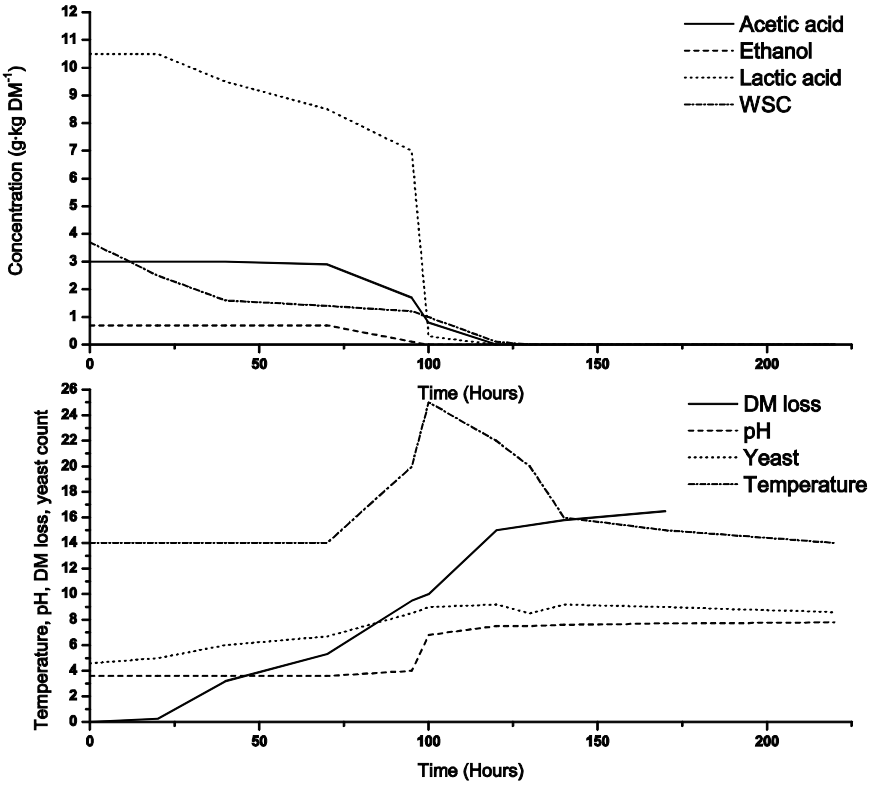

Figure 1.4: Illustration of the theoretical development of pH, dry matter loss (% DM), yeast count (logCFU), temperature (^{o}C), and concentrations (g·kg^{-1}) of water soluble carbohydrates (WSC), lactic acid, acetic acid and ethanol in maize silage during aerobic deterioration. Based on data and graphs from Middelhoven and Balen (1988), O'Kiely and Muck (1992) and Ranjit and Kung (2000).

Improper ensiling

In the case of insufficient ensiling, e.g. low DM content, delayed sealing or insufficient compaction, the fermentation will in most cases not be dominated by LAB and pH will not be lowered sufficiently to obtain stable silage. Populations of Enterobacteria, Clostridia and Bacilli can increase and cause illness in cows, degradation of protein and DM, reduction of silage palatability and increased spore-count in milk decreasing milk quality (Driehuis and Elferink, 2000; McDonald et al., 1991; Wilkinson, 1999). Improperly ensiled crops can also have growth of yeast and filamentous fungi further degrading the product in the same manner as seen during aerobic deterioration.

Antifungal silage additives

Traditionally silage production relies entirely on the natural microbiota of the crop. Therefore large variations occur between years, farms, fields, varieties etc. In order to exert some control over the fermentation different additives have been developed for silage making.

There are different additive components which can effect the fermentation: a) acids to lower the pH of the crop from the beginning of ensiling, b) antimicrobial components controlling the microbial composition, c) microbial inoculants in sufficient amounts to dominate fermentation, d) enzymes to liberate structural carbohydrates for fermentation and e) easily fermentable carbohydrates, e.g.

molasses to ensure high production of lactic acid by LAB. For further information on the types, use and modes of action of silage additives in general see (Kung et al., 2003; McDonald et al., 1991).

The use of silage additives is not very common in Denmark (Wilkinson and Toivonen, 2003), particularly not in maize silage. Maize silage ferments easily and has a low buffer capacity (Lin et al., 1992) so additives to ensure proper ensiling are generally not needed. The Danish Agricultural Advisory Service does not recommend additives for maize silage with less than 35% DM because the economic benefits are doubtful (Nielsen et al., 2009). Weiss (1996) cf. (Allen et al., 2003) reviewed the use of microbial additives in maize silage and did not find substantial evidence for economic benefits on the basis of differences in nutrient composition, pH, lactic acid concentration and DM recovery. Some additives can however improve the aerobic stability of maize silages, especially if the DM content is high.

Some organic acids both acidify the silage and have specific antimicrobial properties which are also used in the food industry. These include formic acid, acetic acid, propionic acid, sorbic acid and benzoic acid (Kung et al., 2003; McDonald et al., 1991; Nielsen and de Boer, 2002). Of particular interest are acetic and propionic acid, as these can be produced by bacteria in the silage (Danner et al., 2003; Filya et al., 2004). A review of experiments using the heterofermentative *Lactobacillus buchneri* as silage additive concluded that this increases the aerobic stability of maize silage (Kleinschmit and Kung, Jr., 2006). *L. buchneri* produces acetic acid by its normal fermentation pathway, and is also shown to convert lactic acid to acetic acid and 1,2-propanediol under anaerobic conditions (Elferink et al., 2001). LAB can also produce other types of antifungal compounds, as reviewed by Schnürer and Magnusson (2005).

1.4 Filamentous fungi in silage

As mentioned earlier well preserved maize silage is a very hostile environment for most fungi. The combination of anaerobic and very acidic conditions prevents their growth. None-the-less growth of filamentous fungi is seen regularly in maize silage stacks (Figure 1.5). This may be the consequence of exposure to atmospheric air i.e. near the surface or next to a hole in the plastic cover. In some cases growth occurs in the middle of what appears to be well preserved silage as was the case with the large hot-spot in the upper left corner of Figure 1.4. The most common species are *Penicillium roqueforti*, *Pen. paneum*, fungi of the class *Zygomycetes*, *Aspergillus fumigatus*, *Monacus ruber*, *Byssochlamys nivea*/*Paecilomyces niveus*, *Geotrichum candidum* and *Fusarium* spp. (Pelhate, 1977; **Paper I**).

Penicillium roqueforti

The most commonly encountered filamentous fungus in silage is *Penicillium roqueforti* as reviewed in (**Paper I**). The closely related *Pen. paneum* is also very common and since the separation of *Pen. roqueforti* into the three taxa *Pen. roqueforti*, *Pen. paneum* and *Pen. carneum* was introduced only in 1996 (Boysen et al.), older reports of *Pen. roqueforti* may refer to either of the three. Among the *Penicillum* species isolated from silages associated with ill-thrift of animals and identified by Boysen et al. (2000) and Sumarah et al. (2005) there where however no *Pen. carneum*.

Figure 1.5: Examples of hot-spots with growth of filamentous fungi in maize silage. Most of the pictures are taken by Birgitte ML Raun.

Pen. roqueforti is able to grow under conditions like those in a silage stack: Growth was observed by van den Tempel and Nielsen (2000) at 10°C on a cheese medium at pH 4.5 in an atmospheres containing 0.3% O_2 and 25% CO_2. Taniwaki et al. (2001) observed growth of *Pen. roqueforti* on cheese in 40% CO_2 and 1% O_2, which was only 11.5% less than in atmospheric air. Optimal pH is between 4 and 5 (Vivier et al., 1992) and it is tolerant to high levels of organic acids like acetic acid and propionic acid (Samson et al., 2002; Suhr and Nielsen, 2004; Vivier et al., 1992). The optimal temperature is 25°C for growth and 20°C for carbon conversion efficiency (Li et al., 2009), but it is capable of growing at 5°C (Frisvad and Samson, 2004).

Zygomycetes

Fungi of the class *Zygomycetes*, order *Mucorales* are also typical spoilage fungi of silage(**Paper I**). They are very wide spread saprotrophic fungi and are commonly found in soil and compost, and as causative agents of rot on fruits, grain and vegetable (Carlile and Watkinson, 1994; Samson et al., 2002). They sporulate profoundly and grow very rapidly (Samson et al., 2002). Some species are known to cause invasive fungal infections in humans and animals (Chayakulkeeree et al., 2006; Jensen et al., 1994). They grow very rapidly and can cover whole Petri dishes within less than a week. Some *Mucorales* are capable of fermentative metabolism and growth under anaerobic conditions (Carlile and Watkinson, 1994). The use of *Rhizopus oligosporus* in fermentation of tempeh together with LAB shows its ability to grow at pH values down to 4.9 (Feng et al., 2005). Le Bars and Escoula (1974) report germination and growth under anaerobic conditions of several zygomycetes isolated from silage. They did however grow as yeast-like cells instead of the normal filamentous structure, a phenomenon which is also mentioned by Carlile and Watkinson (1994). Optimum temperatures vary from 20°C for *Mucor* spp. up to approximately 36°C for *Absidia* spp. (Samson et al., 2002).

Aspergillus fumigatus

Asp. fumigatus is also a common saprobic fungus occurring in soil and compost (Latge, 1999; Samson et al., 2002) and is also often found in silages (**Paper I**). It sporulates abundantly and its spores are very hydrophilic so they are abundant in air (Latge, 1999). It produces at least 226 potentially bioactive secondary metabolites including the highly toxic gliotoxin (Frisvad et al., 2009). It can also cause respiratory and intestinal infections in humans and animals, particularly in immunocompromised individuals (Jensen et al., 1994; Latge, 1999) and thus poses dual risks to exposed individuals. It is a thermotolerant fungus which grows well at 37°C and can tolerate temperatures up to 55°C (Bhabhra and Askew, 2005; Samson et al., 2002). The species is reported to grow at low oxygen tensions (Samson et al., 2002) and there are indications of fermentative metabolism in the species at low oxygen concentrations (1%) (Willger et al., 2009). No sporulation or growth was however observed by Taubitz et al. (2007) under anaerobic conditions. Anaerobic digestion of organic household waste with pH 4.9 at 37 and 55°C reduced the number of *Asp. fumigatus* below the limit of detection (LOD) of 10^2 CFU·g^{-1} (Schnürer and Schnürer, 2006). Temperatures in healthy silage do not reach as high as 37°C and the microbial competition is quite different from waste, so survival of *Asp. fumigatus* spores in silage is possible, but germination and growth is not likely unless O_2 diffuses in the stack. Secondary infection in silage from airborne spores is also a possibility, in particularly if the rate of use of silage is low.

Monascus ruber and *Byssochlamys nivea*

The ascomycetes *M. ruber* and *B. nivea* are also commonly isolated from silage (**Paper I**). In both species the ascospores are heat resistant. *M. ruber* has been isolated from a can of green olives with reduced O_2 level (Panagou et al., 2002) and is able to grow down to a pH below 3 (Panagou et al., 2005) with optimum growth at temperatures around 35°C (Panagou et al., 2003). Similarly *B. nivea* is an important contaminant of canned fruit and fruit-juices where it survives both heat-treatment, low pH and low O_2 levels (Samson et al., 2002). *Paecilomyces niveus*, the anamorphic state of *B. nivea*, is also capable of surviving anaerobic digestion of waste at both 37 and 55°C (Schnürer and Schnürer, 2006). These species are able to survive in silage and can cause fungal deterioration even at low concentrations of O_2, but the exact limits for growth are not known.

Geotrichum candidum

G. candidum is a fungus displaying both yeast-like and filamentous traits (Eliskases-Lechner, 2002; Pottier et al., 2008). It is also often isolated from silage (**Paper I**). On MEA it forms white filamentous colonies and conidia are formed by the breaking up of fertile hyphae into barrel-shaped arthroconidia (Samson et al., 2002). It is very common in e.g. milk, soil, air, water and silage (Pottier et al., 2008; Samson et al., 2002) and is used for cheese making, e.g. camembert, in combination with other yeasts and moulds (Eliskases-Lechner, 2002). It is acid-tolerant, grows between 5-38°C (optimum around 25°C) and has a wide pH-tolerance (optimum 5 to 5.5) (Eliskases-Lechner, 2002). Growth on a cheese medium (pH 4.5) was observed in an atmosphere of 0.3% O_2 and 25% CO_2 (van den Tempel and Nielsen, 2000), so growth even in well fermented maize silage may be possible.

Fusarium

Species of *Fusarium* are ubiquitous pathogens of maize pre-harvest (Placinta et al., 1999; Sørensen, 2009). They are therefore also transferred to the silage stacks, but most *Fusarium* species are generally not able to grow under acidic and anoxic conditions. Mansfield and Kuldau (Mansfield and

Kuldau, 2007) examined maize by culturing and DNA sequence based techniques before and after ensiling and found eight species of *Fusarium* in the newly harvested maize but none in the silage. Only *F. oxysporum* can tolerate anoxic and acidic conditions (Samson et al., 2002). There are reports of *F. oxysporum* being capable of anaerobic growth even though size and morphology was different from colonies grown in atmospheric air (Taniwaki et al., 2009). None-the-less there are reports of various *Fusarium* species isolated from silage (El-Shanawany et al., 2005; Garon et al., 2006; Pereyra et al., 2008b; Reyes-Velazquez et al., 2008; Richard et al., 2007). Examples of the species found are *F. verticillioides, F. oxysporum, F. culmorum, F. equiseti, F. graminearum* and *F. solani*. This can be caused by survival of conidia, improperly fermented silage, re-colonization after (partial) aeration, or possibly more resistant *Fusarium* strains.

The presence of filamentous fungi in maize silage is thus well documented. On the basis of the present knowledge on the physiology of common silage fungi, it is unlikely that growth of filamentous fungi takes place in a well prepared maize silage with a pH below 4, <0.5% O_2 and >60% CO_2. Of the commonly found spoilage fungi in silage *Pen. roqueforti*/*Pen. paneum* and *G. candidum* are those most likely to be capable of growth in a well preserved silage. Growth is however possible for most of the encountered species if just one of the inhibiting parameters fail.

1.5 Post-harvest mycotoxins in maize silage

The growth of filamentous fungi is often accompanied by production of secondary metabolites. In its broadest sense "a fungal secondary metabolite is a chemical compound produced by a limited number of species in a genus, an order, or even a phylum, and has a high differentiation power" (Frisvad et al., 2008). Many of the secondary metabolites can be regarded as mycotoxins, i.e. compounds that in small concentrations can evoke an acute or cronic disease in vertebrate animals when introduced via a natural route (Frisvad and Thrane, 2002). Other secondary metabolites cannot be classified as mycotoxins in this sense, but are biologically active e.g. toxic to insects, antibacterial, antifungal and some have beneficial effects, which are used in the medical world (e.g. monacolins).

Most of the fungi common in maize silage are known to produce several mycotoxins and other secondary metabolites (Table 1.2). The production of secondary metabolites is very dependent on the growth conditions of the fungi. In general, complex growth media with high concentrations of glucose and/or sucrose, yeast or malt extract, minerals and trace metals, result in a higher chemical diversity and higher concentrations of secondary metabolites than poorer media (Frisvad et al., 2008). Most laboratory examinations of fungal metabolic profiles are based on cultures grown on such complex media, often at optimal growth temperatures and under fully aerobic conditions. The fact that a fungus is able to produce a certain secondary metabolite under optimal conditions in the laboratory does not mean that it is capable of doing so in the sugar and oxygen-poor environment of a silage stack. Taniwaki et al. (2009) examined the growth and mycotoxin production of different food spoilage fungi in modified atmospheres, including *Pen. roqueforti* and *B. nivea*. While these two fungi were able to grow in <0.5% O_2 and 20% CO_2, and *B. nivea* also at 40% and 60% CO_2, their production of roquefortine C and patulin, respectively, was greatly reduced.

Metabolic profiles

Table 1.2 lists some of the secondary metabolites produced by *Pen. roqueforti*, *Pen. paneum*, *Asp. fumigatus*, *M. ruber* and *B.nivea/P.* niveus, together with references to those metabolites, which have been detected in grass or maize silage.

The secondary metabolic profiles of *Pen. roqueforti* and *Pen. paneum* are described by Nielsen et al. (2006) and O'Brien et al. (2006). These two penicillia produce many and chemically diverse secondary metabolites. PR-toxin is often emphasized because it has documented toxic effects (Arnold et al., 1978) and has been related to incidents of ill-thrift, disease and abortion in cattle (Seglar et al., 1997 cf. Sumarah et al., 2005; Veselý et al., 1981). Among the most detected secondary metabolites in silage are roquefortine C and mycophenolic acid, which have been detected in concentrations up to 50 and 117 $mg \cdot kg^{-1}$ in silage (**Paper I**). Patulin is also very well known. It is a confirmed mycotoxin (Frisvad et al., 2004) and is produced by *Pen. expansum* and *B. nivea* as well as *Pen. paneum*. Other metabolites include roquefortine A and B, marcfortine A,B and C and andrastin.

The production of secondary metabolites by *Asp. fumigatus* has been reviewed by Frisvad et al. (2009). They reach an impressive count of at least 226 potentially bioactive secondary metabolites, which can be arranged in 24 biosynthetic groups. A selection of these are presented in Table 1.2. Gliotoxin is the best known, due to its long known toxic effects in vertebrates and because it has been found in human and animal tissue infected with *Asp. fumigatus* (aspergillosis) (Frisvad et al., 2009; Latge, 1999). Gliotoxin has been detected in silages and on feed substrates (Boudra and Morgavi, 2005; Pereyra et al., 2008a; Richard et al., 2007) but the general levels of gliotoxin as well as the possible presence of other of the toxic *A. fumigatus* metabolites in silages still need to be elucidated.

Table 1.2: The most common fungal post-harvest contaminants of silage, some known secondary metabolites and secondary metabolites confirmed in silage. Adapted from (Paper I).

Species	Secondary metabolites	Detected in silage[a]	Ref.
Penicillium roqueforti[b]	Agroclavine	+	(O'Brien et al., 2006)
	Eremofortin C		
	Mycophenolic acid	+, 1.3, 35, 117	(Mansfield et al., 2008; Nielsen, Unpublished; O'Brien et al., 2006; Schneweis et al., 2000)
	PR-toxin	+	(Nielsen, Unpublished)
	PR-amide and PR-imine		
Penicillium roqueforti and	Roquefortine A,D + 16-OH-roquefortine	+	(O'Brien et al., 2006)
	Roquefortine C	+, 5.7, 36, 50	(Auerbach et al., 1998; Mansfield et al., 2008; Nielsen, Unpublished; O'Brien et al., 2006)
Penicillium paneum[b]	Andrastin A, B and C	+	(Nielsen, Unpublished; O'Brien et al., 2006)
	Citreoisocoumarin	+	(O'Brien et al., 2006)

Species	Secondary metabolites	Detected in silage[a]	Ref.
	Orsellinic acid		
	Festuclavine	+	(O'Brien et al., 2006)
Penicillium paneum[b]	Marcfortine A	+	(O'Brien et al., 2006)
	Marcfortine B and C		
	Patulin	1.2, 40	(Escoula, 1974; Mansfield et al., 2008)
	Gentisic acid		
Aspergillus fumigatus[c]	Gliotoxin	0.878, 6.50	(Pereyra et al., 2008a; Richard et al., 2007)
	bis-dethio-bis(methylthio)-gliotoxin	+	(Nielsen, Unpublished)
	Fumigatins		
	Trypacidins		
	Sphingofungins		
	Pseurotins		
	Helvolic Acid		
	Fumagillins		
	Fumigaclavines		
	Fumitremorgines		
	Diketopiperazines		
	Fumiquinazolines		
Byssochlamys nivea/ Paecilomyces niveus	Patulin	1.2, 40	(Escoula, 1974; Mansfield et al., 2008)
	Byssochlamic acid		
	Mycophenolic acid	+, 1.3, 35, 117	(Mansfield et al., 2008; Nielsen, Unpublished; O'Brien et al., 2006; Schneweis et al., 2000)
Monascus ruber	Citrinin	0.037, 0.064, 0.25	(Garon et al., 2006; Richard et al., 2007; Schneweis et al., 2001)
	Monacolins	65.4	(Schneweis et al., 2001)
	Pigments e.g. ankaflavin		
	Monascopyridines		
Zygomycetes	Rhizoxins		
	Rhizonin A and B		
Geotrichum candidum	2-hydroxy-3-phenylpropanoic acid		

[a] +: metabolite detected in silage samples. Numbers state max. concentrations in mg kg^{-1} were quantitative determination has been performed.
[b] Based on (Nielsen et al., 2006; O'Brien et al., 2006)
[c] 226 extrolites registered by Frisvad et al. (2009)

The list of secondary metabolites from *M. ruber* and *B. nivea* is much shorter with citrinin, the monacolins, patulin and mycophenolic acid as the most well known.

In general , the *Zygomycetes* are not known to produce many secondary metabolites (Frisvad et al., 2008). The biologically active rhizoxins and the toxic rhizonins A and B have been reported from *Rhizopus microsporus* (Jennessen et al., 2005) but have later been shown to originate from an endosymbiotic bacteria (Partida-Martinez et al., 2007; Partida-Martinez and Hertweck, 2005). Thus *Zygomycetes* can, at least indirectly, lead to mycotoxin contamination of food and feed, but it is not known if *Zygomycetes* have caused mycotoxicosis (Frisvad and Thrane, 2002). They can however cause invasive infections (zygomycosis) in humans and animals (Chayakulkeeree et al., 2006; Jensen et al., 1994), particularly in immunocompromised individuals.

Geotrichum candidum is not known to produce any mycotoxins, but it has been described as a weak pathogen of plants, animals and humans (Eliskases-Lechner, 2002). A safety-assessment of the fungus (Pottier et al., 2008) states that no food-borne disease has been linked to the consumption of products containing *G. candidum* and that cases of infections caused by this fungus are very rare (<1 per year). The risk of direct toxic effects of *G. candidum* in silage therefore seems very small, whereas this fungus is known to produce antibiotic and antifungal compounds (Dieuleveux et al., 1998; Tariq and Campbell, 1991). It may also reduce the palatability of silage (Pelhate, 1977), thereby reducing feed-intake by livestock.

It is clear that fungi growing in maize silage are capable of producing a wide range of mycotoxins and other secondary metabolites. In addition to the above mentioned compounds, fungal secondary metabolites produced pre-harvest e.g. by species of *Fusarium*, *Alternaria*, *Phoma* and *Aspergillus* can also be present in silage (Sørensen, 2009). There may also be yet unknown compounds produced in silage. *Asp. fumigatus* is a very comprehensively examined species because it is a human pathogen. This may be one of the explanations of why its metabolic profile is so comprehensive. Further investigations of the other species may reveal new secondary metabolites. There is thus a need for analytical methods for the detection of mycotoxins in silage samples in order to evaluate the extent and severity of mycotoxins contamination of silage.

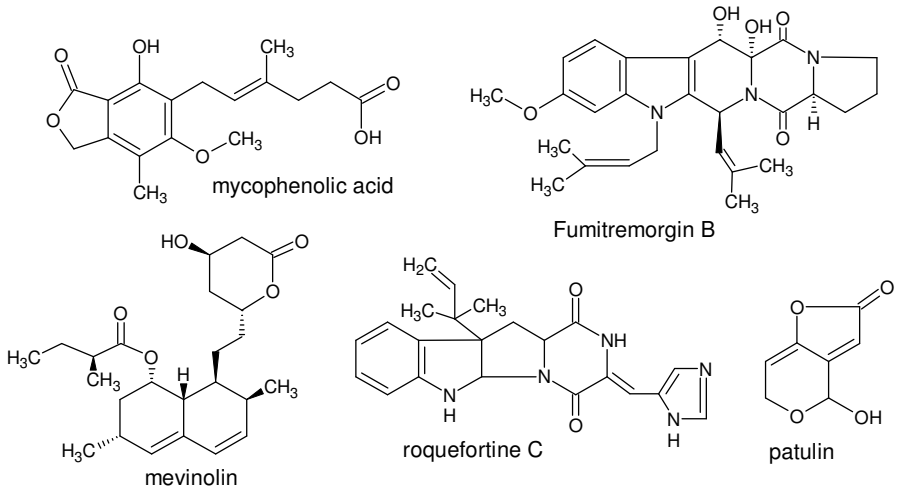

Figure 1.6: Examples of fungal secondary metabolites from known fungal contaminants of silage.

Methods of mycotoxin analysis

Any method of extraction and chemical analysis of fungal metabolites invariably includes selective steps. This can improve the detection of some compounds but also mask the presence of others. The chemical structures in Figure 1.6 illustrate the chemical diversity displayed by fungal metabolites from silage contaminants. There are highly polar compounds like patulin and relatively apolar compounds like mevinolin and fumitremorgin B. Some are organic acids (e.g. mycophenolic acid) while others are alkaloids (e.g. roquefortine C). It is thus clear that it is difficult to selectively extract all of these different compounds at the same time.

Many methods covering one or a few mycotoxins have been published. Methods and developments within the field of mycotoxin analysis have been reviewed by Zöllner and Mayer-Helm (2006), Krska et al. (2008) and Cigic and Prosen (2009). They all highlight the use of tandem mass spectrometry

Table 1.3: Characteristics of known methods for multi mycotoxin detection in silage samples.

References	Mycotoxins	Matrix	Method
Driehuis et al. 2008	Aflatoxin B1, B2, G1, G2 deoxynivalenol (DON) 3-acetyl-DON 15-acetyl-DON diacetoxyscirpenol ergotamin fumonisin B1, B2 fusarenon-X ochratoxin A mycophenolic acid penicillic acid roquefortine C sterigmatocystin T-2 toxin HT-2 toxin zearalenone	Maize, grass and wheat silage	-Extraction with MeCN:water 80:20 (v/v). -No clean-up -Reverse phase LC-MS/MS analysis
Garon et al 2006 Richard et al. 2007 Richard et al. 2009	aflatoxin B1 citrinin deoxynivalenol fumonisin B1 gliotoxin ochratoxin A zearalenone	maize silage	-Extraction with MeOH:water 80:20 (v/v) -Clean-up on reverse phase SPE columns -Reverse phase LC-MS analysis
Mansfield and Kuldau 2008 (modified from Rundberget and Wilkins 2002)	cyclopiazonic acid patulin mycophenolic acid roquefortine C	maize silage	-Extraction with MeCN:water (9:1 v/v) with 0.1% formic acid -Defatting with hexane -Reverse phase LC-MS analysis
O'Brien et al. 2006	penicillic acid mycophenolic acid roquefortine C patulin roquefortine A[a] andrastine A[a] marcfortine A[a] festuclavine[a]	grass silage	-Extraction with ethyl acetate -Clean-up on reverse phase SPE columns -Reverse phase LC-MS analysis

[a]detected in silage samples but not validated

(MS/MS) as one of the major break-troughs in modern mycotoxins analysis. Because of the selective detection of the MS/MS the need for laborious, time-consuming and selective clean-up is highly reduced. In many cases raw extracts of food or feed are used without any clean-up.

The detection of multiple mycotoxins in silage samples has been performed. A list of methods used are presented in Table 1.3. As seen in the table the methods by Driehuis et al. (2008) and Garon et al. (2006) only include very few post-harvest toxins while the studies by Mansfield and Kuldau (2008) and O'Brien et al. (2006) cover metabolites from *Pen. roqueforti* and *Pen. paneum*.

Other methods of interest include the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of 87 fungal analytes by Sulyok et al. (2007). It has been validated for a matrix of bread crumbs and applied on samples of mouldy food, so the performance on silage samples is not known. Another multi-method for the detection of 31 selected fungal metabolites has been published recently (Kokkonen and Jestoi, 2008). It employs Accelerated Solvent Extraction on samples of wheat, barley and oat grain. The effect on silage samples is therefore not known and the method only includes a few compounds attributable to the common fungal post-harvest contaminants of silage.

A particular concern in the development of methods for mycotoxins detection and quantification is the availability of reference standards. Many hundreds of fungal secondary compounds are known but very few can be bought as pure reference compounds. The availability is focused on the known toxic compounds covered in regulations (e.g. aflatoxins, trichothecenes, fumonisins, ochratoxin a, patulin) as well as compounds such as mycophenolic acid due to its pharmaceutical application as an immunosuppressive drug, and citrinin and gliotoxin due to their potent toxic effects.

Toxicological aspects

With many and very different mycotoxins as possible contaminants of maize silage it is clear that the possible toxicological effects caused by post-harvest infection in silage are numerous. A comprehensive overview of the biological activity and toxicology of the relevant fungal secondary metabolites is beyond the scope of this project. To illustrate the complexity of the subject some examples of fungal secondary metabolites from post-harvest contaminants and their documented biological activities are mentioned in Table 1.4.

Most of the toxic effects are however documented in mice, rats or other mono-gastric animals. For the majority of mycotoxins, interactions between rumen microorganisms and the fungal secondary metabolites are unknown. It is generally accepted that ruminants are less susceptible to mycotoxins than other animal species (Fink-Gremmels, 2008a; Hussein and Brasel, 2001; **Paper I**; Yiannikouris and Jouany, 2002), which is attributed to the microbial activity in the rumen. Known examples of metabolic conversions of mycotoxins in ruminants are described in several reviews. Conversions to less toxic substances includes transformation of ochratoxin A to ochratoxin α and deoxynivalenol (DON) to de-epoxy nivalenol (DOM)(Fink-Gremmels, 2008a; Hussein and Brasel, 2001). Metabolisation may also lead to compounds with different but not less potent toxicity e.g. zearalenone to α-zearalenol and aflatoxins to aflatoxicol and aflatoxin M_1 (Fink-Gremmels, 2008a; Yiannikouris and Jouany, 2002). Aflatoxin M1 is partly excreted in milk (e.g. (Fink-Gremmels, 2008a; Yiannikouris and Jouany, 2002). Conjugated mycotoxins (Berthiller et al., 2005) could also be released in the rumen, if they are present in the silage.

Another form of interaction is the effects of mycotoxins on the rumen microbial system. Patulin and extracts of *Asp. fumigatus* have been found to affect rumen fermentation in vitro (Morgavi et al., 2003; Morgavi et al., 2004).Other compounds with antimicrobial effects could also affect the microbial ecosystem of the rumen and thereby the efficiency of ruminant digestion. In a feeding experiment with multi-catheterised cows, Kristensen et al. (2007) detected changes in the rumen fermentation after feeding with *Penicillium* and *Fusarium* toxin contaminated maize silage for 14 days. Alterations in the ruminal protein utilisation were also detected by Dänicke et al. (2005) after feeding with *Fusarium* toxin contaminated wheat. There were no effects on the overall performance of the cattle in either of the experiments. It is possible that impairment of the digestion in high-yielding dairy cows may lead to a general weakening of the animal. The transition period around calving constitutes a particularly high physiological stress (Fink-Gremmels, 2008b). Alterations in the rumen function may also affect the metabolism of other mycotoxins.

The presence of immunosuppressive compounds may also affect live-stock. This subject has been reviewed by Oswald et al. (2005) and Bondy and Pestka (2000) but again specific knowledge on ruminants is limited. Feeding trials where sheep were fed the known immunosuppressant mycophenolic acid revealed effects on the morphology of immune organs and the expression of certain immune enzymes (Baum et al., 2005; Dzidic et al., 2006). No effects on the overall health status of the sheep were however observed in either of the trials even though the doses of MPA were high compared to what silage has been seen to contain.

Such trials or other laboratory examinations of toxicity are usually done under simplified conditions, e.g. with only one contaminant or a few related compounds. In naturally infected feedstuffs there may easily be several different mycotoxins present and synergistic effects can take place. Synergistic

Table 1.4: Examples of biological activities of fungal secondary metabolites from the common post-harvest contaminants of silage Penicillium roqueforti, Pen. paneum, Aspergillus fumigatus, Byssochlamys nivea and Monascus ruber. Based on information compiled by (Boudra and Morgavi, 2005; Fink-Gremmels, 2008b; Frisvad et al., 2009; Frisvad and Samson, 2004; Nielsen et al., 2006; O'Brien et al., 2006; Paper I).

	acute toxic	cytotoxic	neurotoxic	immunosuppressive	nephrotoxic	carcinogenic	mutagenic	tremorgenic	antimicrobial	nematicidic
Roquefortines		x	x						x	
PR-toxin	x					x	x			
Patulin		x	x	x	x				x	
Mycophenolic acid				x					x	
Gliotoxin		x		x			x			
Citrinin					x				x	
Marcfortines										x
Fumitremorgins								x		

effects between *Fusarium* toxins are mentioned by Yiannikouris and Jouany (2002). Of specific interest for cattle fed silage Morgavi et al. (2004) did not find a pronounced effect of pure gliotoxin on *in vitro* rumen fermentation. A very high concentration of 80 $\mu g \cdot ml^{-1}$ was necessary to affect fermentation pattern. But a pronounced effect was seen when an *Asp. fumigatus* extract containing only 8.8 $\mu g \cdot ml^{-1}$ gliotoxin besides other fungal secondary metabolites, was added to the rumen fermentors.

Actual cases of mycotoxin intoxications in cattle are rare and by nature they are difficult to prove. Frequent symptoms of mycotoxicosis are reduced feed consumption, decreased animal performance and/or reproductive problems which may also be ascribed to many other causes (Fink-Gremmels, 2008a; Wilkinson, 1999; Yiannikouris and Jouany, 2002). Some case studies where mouldy silage has been associated with disease in dairy cattle are published (Cole et al., 1977; Gerisch et al., 1981; Lloyd, 1980; Veselý et al., 1981). In all four cases visibly mouldy silage had been fed to the cattle. In 3 studies (Cole et al., 1977; Gerisch et al., 1981; Veselý et al., 1981) mycotoxigenic fungi were isolated and shown to produce mycotoxins in culture. Gerisch et al. (1981) did not identify the toxin but established the connection between symptoms and infected silage by feeding it to healthy animals, which became ill. Cole et al. (1977) tested culture extracts of the isolated *Asp. fumigatus* on calves. The study by Lloyd (1980) detected citrinin and ochratoxin in the silages combined with a decrease in symptoms when the feeding of infected silage was discontinued.

These examples illustrate the difficulty of determining a connection between mouldy silage and intoxication in live-stock. Unless it is caused by a single mycotoxin which can be isolated from the feed, from the fungus *in vitro* and which has proven toxic effects on ruminants, the connection is dubious. If the symptoms are caused by a combination of mycotoxins, the only proof of a connection to the feed may be feeding of the suspected product to healthy animals, which is ethically questionable. Furthermore this approach is only useful for fast acting toxins. The effects of chronic low dose exposure are not detected. Such studies probably require long-term experiments with high-yielding cows under normal farm conditions to detect possible adverse effects. In real-life cases the silage in question may be long gone. The investigation of synergistic and long-term effects is gaining more and more interest in health and environmental sciences. More research within this field is also required for a better understanding of the possible effects of mycotoxins.

2 The present study

2.1 Background

The production of maize silage has been practised in warmer parts of the world for decades and knowledge on fungal spoilage under these conditions has been gathered. With the development of short season maize hybrids, the production of maize silage has spread to new geographical areas in the north of Europe and North America. Cows are often fed the same ration all year round in modern dairy farming, so maize silage is now stored for 12-14 months or more.

Growth of filamentous fungi takes place in maize silage and causes spoilage of the feed. This constitutes an economic loss for the farmer and can have detrimental effects on the health of live-stock, farmers and possibly consumers of meat and dairy products. Very little information is available on the mycobiota of maize silage beyond the initial 6-8 months of ensilage.

Comparison of the existing reports on the mycobiota of silage indicates variations in numbers and species of filamentous fungi between different regions of the world. This may be due to variations in e.g. climate, crop varieties and methods of ensiling. As a consequence these reports may not reflect the situation in present day Danish maize silages.

In 2003 and 2004 several cases of ill-thrift, disease and death of dairy cows were suspected of being caused by mycotoxins from maize silage (Houmann, 2003c; Houmann, 2004; Woller, 2004). The cases were not unambiguously confirmed as mycotoxin related, but they lead to an intense debate about the risks of both pre- and postharvest mycotoxins in maize silage and other possible reasons for the observed cases (Houmann, 2003a; Houmann, 2003b; Houmann, 2003d; Jørgensen et al., 2004; Jørgensen, 2005; Mortensen, 2003). This emphasised the need for more information on the occurrence of filamentous fungi and mycotoxin in maize silages produced and used at modern Danish dairy farms.

2.2 Aims of the study

The overall aims of this study are to explore:

- Which fungi are present in Danish maize silages?
- Under what conditions do filamentous fungi proliferate in and spoil Danish maize silage?
- Which mycotoxins are produced post-harvest by these fungi in Danish maize silages?

In order to do this a series of specific studies and experiments have been conducted:

- A study of the microbial dynamics of maize silage stacks during whole-season storage (chapter 3.1 and **Paper II)**
    - A survey of 20 maize silage stacks over a period from 3 to 11 months after ensilage. It documents the fungal species present in Danish maize silages and explores the correlation of their occurrence with physical and chemical properties of the silages as well as with other microbial factors and the storage time of the silage
- Determination of the microbial heterogeneity of maize silage stacks and its implications for silage sampling (Chapter 3.2 and **Paper III**)

- This study evaluates the uncertainties associated with sampling silage for quantification of fungal contaminants. It provides useful information for the interpretation of the results of **Paper II** and for the general interpretation of reports on the microbial state of a silage stack

- Measurement of oxygen, carbon dioxide and temperature in maize silage stacks *in situ* (Chapter 3.3 and **Paper IV**)
    - Oxygen concentration, carbon dioxide concentration and temperature are three properties of silage which may be closely linked to the occurrence and growth of filamentous fungi in silage. Existing methods for determination of these properties are very difficult to apply to full scale maize silage stacks. Tests were conducted of new methods suitable for *in situ* detection of oxygen and carbon dioxide concentrations and temperature variations in silage stacks
- Development of an LC-MS/MS method for the detection of 27 fungal secondary metabolites in maize silage samples (chapter 3.4 and appendix B)
    - To be able to determine whether mycotoxins are present in maize silage samples a new multi-mycotoxin method was required. A quick and simple method of extraction of silage samples followed by LS-MS/MS analysis has been developed and tested for 27 fungal secondary metabolites. Included are mycotoxins typical of the most common pre- and post-harvest fungi in maize and maize silage as well as other secondary metabolites of interest

Chapter 3.1 to 3.4 summarise the conducted studies and their results, with references to original manuscripts where applicable.

2.3 Delimitations

This study is part of a multi-institutional program containing several research projects with a high degree of collaboration. With this in mind it should be highlighted that the present study is focussed on the occurrence of filamentous fungi in maize silage post-harvest, whereas growth of filamentous fungi pre-harvest and the presence of pre-harvest mycotoxins in silage are covered by the PhD thesis of Jens L. Sørensen (2009). Similarly, the toxicological perspectives of mycotoxin in maize silage are presently being explored by PhD student Rie R. Rasmussen at The National Food Institute, Technical University of Denmark and is therefore not part of the present PhD thesis. Nutritional aspects for the dairy cows are being explored at Department of Animal Health and Bioscience, Faculty of Agricultural Sciences, Aarhus University.

3 Experiments and results

3.1 Microbial dynamics in maize silage stacks during whole-season storage

*This chapter contains a brief presentation of the study and its main results based on **Paper II**. For detailed descriptions of methods etc. see **Paper II**.*

Introduction

The dynamics of the microbiology of silage of different crops has been studied extensively for the first days, weeks and months after ensiling (McDonald et al., 1991; McEniry et al., 2008a; Middelhoven and Baalen, 1988; Naoki and Yuji, 2008) giving a good understanding of the principles of ensiling. However, to our knowledge only very few studies of silage mycobiota include silage more than 6 months old (Garon et al., 2006; Reyes-Velazquez et al., 2008; Richard et al., 2007) and none of those are surveys. With the changes in agricultural practices towards all-year feeding of silage, silage can be older than 12-14 months at the time of feeding. A better understanding of the long-term dynamics of silage is therefore important to optimize long term storage, minimize fungal deterioration and decrease the risk of mycotoxins in silages.

The goals of this study are to monitor the seasonal variations in the microbiology of maize silage and determine whether the risk of fungal spoilage and contamination of maize silage *in situ* varies over a whole season. This was done by a) determining the fungal species present in maize silage stacks used for feed-out at 20 selected farms from 3 to 11 months after ensiling, b) determining whether the number of viable microorganisms in silages varied during this period and c) examining whether microbial variations between stacks and over time correlated with physical and chemical parameters of the silage.

Method

Maize silage stacks at 20 dairy farms were visited 5 times at two month intervals from January to September 2007. During each visit a silage sample was collected in full depth of the silage stack approximately one meter behind the cutting face. Hot-spots of fungal growth visible from the cutting face were also collected. The numbers of colony forming units of filamentous fungi (on the media V8 juice agar (V8) and dichloran glycerol 18% agar (DG18)), yeasts (on the medium malt yeast glucose peptone agar (MYGP)) and lactic acid bacteria (on the medium MRS) were assessed at each sampling time-point. The culturable species of filamentous fungi in both healthy looking and visibly

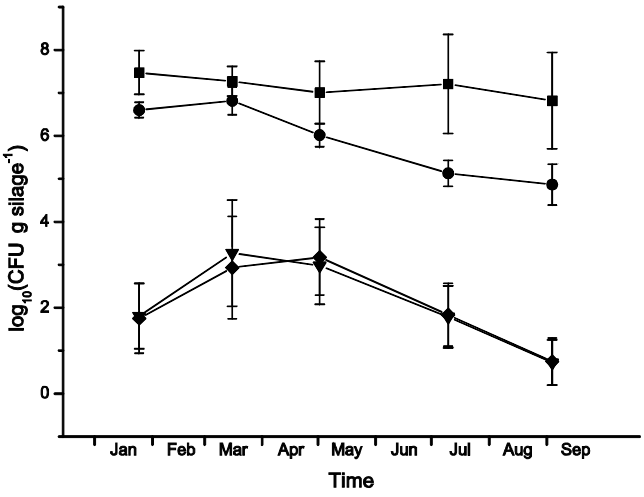

Figure 3.1: Average of log_{10} transformed counts of colony forming units of filamentous fungi on V8 (▼) and DG18(♦), lactic acid bacteria (■) and yeasts (●) at approx. 3,5,7,9, and 11 months after ensiling. Error bars indicate 95% confidence intervals.

mouldy samples were isolated and identified. The correlation between microbial CFU counts and time after ensilage as well as with 16 physical and chemical properties of the silage samples was tested.

Results

During the 8 months sampling period from 3 to 11 months after ensilage there were significant changes in the number of CFUs of filamentous fungi found on V8 and DG18, of yeast and of LAB (P=0.0005, P=0.0003, P<0.0001 and P=0.0016, respectively). LogCFU of filamentous fungi and yeast were highest in spring 5 to 7 months after ensiling and lowest in September 11 months after ensiling (Figure 3.1). Fewer hot-spots were also observed and collected in September.

Filamentous fungi were isolated from all farms at all sampling times. The most commonly isolated filamentous fungi were *Penicillium roqueforti*, species of *Zygomycetes* (primarily *Mucor* spp.), *Pen. paneum*, and *Aspergillus fumigatus* (Table x.x). The same species also dominated in the collected hot-spots of fungal growth (Table x.x). Less frequent species were *Geotrichum candidum*, *Byssochlamys nivea*, *Coelomycetes*, *Monascus ruber* and species of *Penicillium* (other than *Pen. roqueforti* and *Pen. paneum*). Other species rarely encountered included *Trichoderma* spp., *Eurotium*

Table 3.1: Number of sampled maize silage stacks from which specific species or groups of filamentous fungi have been isolated at the given sampling time.

	Month				
	Jan (n=20)	**Mar** (n=20)	**May** (n=20)	**Jul** (n=20)	**Sep** (n=18)
Penicillium roqueforti	17	20	18	15	12
Zygomycetes	7	15	14	16	9
Penicillium paneum	11	13	8	10	5
Aspergillus fumigatus	3	7	12	4	2
Geotrichum candidum	2	8	4	5	4
Byssochlamys nivea	4	3	2	5	4
Coelomycetes	2	4	4	3	3
Penicillium spp., other	0	4	0	0	4
Monascus ruber	5	1	0	0	1
Others	10	4	3	13	13

Table 3.2: Number of sampled maize silage stacks containing visible fungal hot-spots with specific species or groups of filamentous fungi as primary contaminant, displayed by sampling time.

	Month				
	Jan (n=20)	**Mar** (n=20)	**May** (n=20)	**Jul** (n=20)	**Sep** (n=18)
Penicillium roqueforti	9	10	11	5	2
Zygomycetes	5	7	10	14	7
Penicillium paneum	7	5	3	2	2
Aspergillus fumigatus	2	6	6	3	1
Geotrichum candidum	0	3	1	1	1
Byssochlamys nivea	3	0	2	3	1
Coelomycetes	2	0	3	2	0
Penicillium spp., other	0	0	0	1	1
Monascus ruber	4	0	0	0	1
Others	1	2	4	5	1
Total	33	33	40	35	17

spp., *Fusarium* spp., one *Cladosporium* sp., one *Aspergillus flavus* and one *Asp. niger*. Other species also includes isolates which could not be identified due to lack of sporulation. They accounted for 9.6% of the total number of isolates and were most abundant in the July and September samples.

The occurrence of *Pen. roqueforti*, *Pen.paneum* and *Asp. fumigatus* followed the same trend as the overall CFU of filamentous fungi and peaked in the March and May samples. *Mucor* species and the other *Zygomycetes* were less frequent than the previously mentioned species except in July, when they were the most frequently isolated group as well as the most frequent contaminants of hot-spots, and in September when they dominated the mycobiota of the collected hot-spots.

Very few *Fusarium* spp. were isolated but the very common *Fusarium* toxin DON was present in all 20 samples from January with an average concentration of 1056 $\mu g \cdot kg^{-1}$ (**Paper II**). T-2, HT-2 and fusarenone-X was present in some of the analysed samples but 3-acetyl DON was not detected in any.

No apparent connection between the occurrence of filamentous fungi and any of the other microbial data or physical/chemical data was seen, except for correlation between the counts of filamentous fungi on V8 and DG18. PLS regression between yeast counts (logMYGP) and the physical/chemical parameters (Raun, B. M. L. and Kristensen, N. B.) gave a positive correlation between the yeast count and the concentrations of ethanol and glucose and negative correlation with ammonia and temperature, but with the optimal number of two principal components the model was not capable of explaining more than 49 and 40% of the variation in the Y and X data, respectively. For the counts of LAB, filamentous fungi on V8 and DG18 the percentages of explained Y-variance were 45, 22 and 17%, respectively.

One difference was also observed between the microbiology of the two types of silage stacks in the experiment: field stacks and planar silo stacks. Nine and 11 months after ensilage the yeast counts were significantly lower in the silo stacks than in the field stacks ($P<0.001$).

Conclusions

The most abundant toxigenic mould species were *Penicillium roqueforti*, *Pen. paneum* and *Aspergillus fumigatus*. It was concluded that extended storage time of maize silage is associated with significant changes in the microbiota of the silage. Filamentous fungi were ubiquitously present in the sampled maize silages but the number of culturable fungal propagules was highest 5-7 months after ensilage and lowest after 11 months. Thereby the risk of fungal spoilage upon aeration is higher after 5-7 months than after 11 months of storage. There were no apparent correlations between the number of cultivable microorganisms and the temperature or chemical composition of the examined maize silages.

3.2 Microbial heterogeneity of maize silage stacks

*This chapter contains a brief presentation of the study and its main results based on **Paper III**. For detailed descriptions of methods etc. see **Paper III**.*

Introduction

Silage stacks represent the classical sampling problem: They contain hundreds of tons of silage but only a few kilogram of silage can be sampled for analysis. Furthermore, the final analysis may only require a few grams of silage. Therefore it is crucial that sampling from silage stacks is done carefully taking into account the unavoidable inhomogeneity of the silage stack to ensure that the results obtained are representative of a larger volume of the silage.

To supplement a larger study of fungal spoilage of maize silage (**Paper II**), repeated sampling was conducted on 5 maize silage stacks to study the intra stack variation of microbial and feed value parameters. Individual samples and sub-samples were analysed for contents of microorganisms and feed value. The purpose of the study was to determine whether variations within silage stacks have a significant impact on the overall measurement uncertainty of individual silage sample. The microbial analysis variance originating from individual steps of the analysis procedure was also evaluated. On the basis of these estimates of variance the number of samples from one stack which is required to obtain predetermined confidence intervals for stack means was calculated.

Method

Five planar silos of maize silage at five different farms (Level Farm in Figure 3.2) were visited. The setup for each farm is illustrated in Figure 3.2. From each stack 5 primary samples were taken in full depth of the maize silage (Level 1 in Figure 3.2). Each of the primary samples were subdivided further (Level 2 and 3-4 in Figure 3.2) to obtain samples of 40 g, suitable for microbial enumeration by serial dilution (Level 5 in Figure 3.2). Plating on Petri dishes was performed in replicate for all serial dilutions (Level 6 in Figure 3.2). Enumeration of colony forming units was performed for filamentous fungi on V8 and DG18, yeasts on MYGP and lactic acid bacteria on MRS. Repeats were performed at all levels to assess the variance components of the method. For level 2, 3-4 and 5 one randomly chosen sub-sample from each farm was chosen for replication, totalling 5 farms, 25 samples at Level 1, 30 sub-samples at Level 2, 35 smaller sub-samples at Level 3-4, 40 dilution series at Level 5 and 80 Petri dish series (on each of the 4 media) at Level 6. Feed value was measured by Near Infrared spectroscopy (NIR) on the 25 samples from Level 1, 5 from each stack.

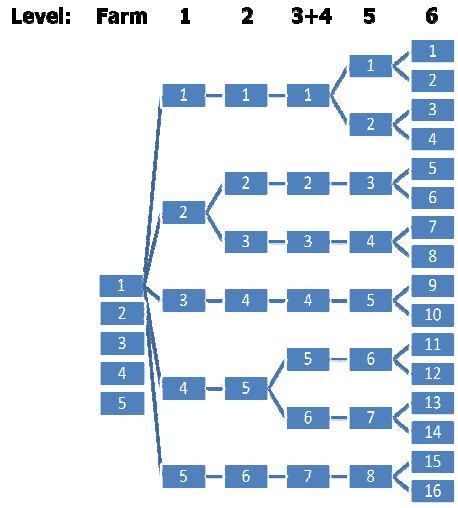

Figure 3.2: Illustration of the sampling plan at each of the 5 farms in the experiment. At each farm 5 primary samples were taken (Level 1) and each of these were further divided by sub-sampling and serial dilution (level 2, 3-4 and 5) with one random replication of the division procedure at each level. Duplicate plating of each serial dilution was performed (Level 6).

Table 3.3: Summary data and standard deviations for the log-10 transformed counts of colony forming units of yeasts on MYGP and lactic acid bacteria on MRS detected in maize silage samples. Five samples were taken from each of 5 stacks and repeats conducted at each subsequent level of the sample reduction procedure (L2-L6). Standard deviation for levels with significant effect on the total variation are included.

	n[a]	mean	S_{stack}	S_{L1}	S_{L2}	S_{L3-4}	S_{L5}	S_{L6}	S_{total}
Yeast	76	4.8	1.44	0.95	ns[b]	0.17	ns[b]	0.06	1.73
LAB	80	7.3	0.50	0.31	ns[b]	0.08	ns[b]	0.05	0.60

[a]Counts below logCFU=2 were not detectable.
[b]ns=non significant

Table 3.4: Summary data and standard deviations for the log-10 transformed counts of colony forming units of filamentous fungi in stack no. 3 enumerated on the media V8 and DG18. Standard deviations for levels with significant effect on the total variation are included.

	n[a]	mean	S_{L1}	S_{L2}	S_{L3-4}	S_{L5}	S_{L6}	S_{total}
V8	14	4.2	1.5	ns[b]	0.25	ns[b]	0.14	1.5
DG18	15	3.6	1.4	ns[b]	0.16	ns[b]	0.06	1.4

[a]Counts below logCFU=2 were not detectable.
[b]ns=non significant

Results

The fungal parameters were highly heterogenously distributed within silage stacks. Relative intra-stack standard deviations were 36, 39 and 20% for filamentous fungi on V8, filamentous fungi on DG18 and yeast, respectively. The calculated numbers of samples needed to determine mean values within 95% confidence intervals of ±1 logCFU were 11, 11 and 7, respectively. LAB were more homogenously distributed with a RSD_{intra} of 8%.

For all microbial parameters the variations between farms (level farm), primary samples (level 1), tertiary/quaternary samples (level 3+4) and dilution series (level 6) were significant in the statistical model (Table 3.3 and Table 3.4).

The feed value parameters were homogeneously dispersed within silage stacks compared to the fungi with values of RSD_{intra} from 1-11%. For pH, lactic acid (g·kg DM^{-1}) and acetic acid (g·kg DM^{-1}) the relative standard deviations between samples from the same stack were 2, 5 and 11% respectively. Results, discussion and conclusions on this topic are presented in **Paper III**.

3.3 Oxygen, carbon dioxide and temperature measurements *in situ*

*Parts of this chapter are also described in **Paper IV**.*

The atmospheric composition inside a silage stack is one of the important factors controlling growth of filamentous fungi. Therefore it is desirable to be able to measure the concentrations particularly of oxygen and carbon dioxide inside maize silage stacks *in situ*.

Previous experiments in this area have involved placing pipes and hoses in silage stacks before fermentation (See introduction to **Paper IV** and Weinberg and Ashbell, 1994) or in the case of big bales of silage the insertion of sampling devices after ensiling (Forristal *et al.*, 1999). These methods may affect the fermentation so the results do not represent naturally fermented silage. Furthermore, if the air samples are collected in sealed containers and brought to the laboratory for analysis it introduces a risk of intrusion of atmospheric air during sampling and transport, which would cause erroneous results.

Temperature is also interesting to monitor in silage stacks from two perspectives. The general temperature levels in silage may affect the silage microbiota by promoting or inhibiting certain groups of microorganisms (Lindgren et al., 1985b; Middelhoven et al., 1990). Furthermore, increased microbial activity in connection with fungal growth in silage releases heat. Monitoring of silage temperature can therefore reveal fungal growth inside sealed silage stacks.

Methods

Two different methods for *in situ* detection of A) O_2 and CO_2 and B) temperature and O_2 in maize silage stacks have been tested.

A) The first method employed a hand-held gas detector fitted with an electrochemical oxygen sensor and an infrared carbon dioxide sensor. The sensors were capable of measuring 0-25% (v/v) of the two gasses. Through a steel-probe gas measurements could be performed from the bottom of drilled holes in silage stacks. A pump inside the detector extracted air for the measurement. Details on the procedure are included in Appendix A.

The gas detector was tested at two full size maize silage stacks located at Gjorslev Gods (GG) and Freerslev Kotel I/S (FK), Zealand, Denmark. Both stacks were planar silos with concrete bottom and 3 meter high concrete sides. They were visited 5 times each at approximately 2 month intervals from January to October 2007. During each visit three measurements were performed at random places within the same approximately 4x4 m area of the silage stacks.

Figure 3.3: Sampling and gas measurement of a maize silage stack at Freerslev Kotel I/S, Gørløse, Denmark.

B) The second method tested was a wireless temperature and oxygen sensor for silage, developed by PhD student Ole Green, Department of Agricultural Engineering, Aarhus University, Denmark

(**Paper IV**). Three units of a proto-type of the sensor were tested in a full-scale maize silage stack located at Gjorslev Gods, Denmark. The dimensions of the silage stack were 10 m × 50 m × 3 m. During silage stack preparation, two sensor units (identified as A25 and B25) were placed inside the stack at a depth of 25 cm, and a third sensor unit (A50) was placed at a depth of 50 cm. A25 and A50 contained temperature sensors, while B25 contained both a temperature and an oxygen sensor. Measurement locations relatively close to the surface (25 and 50 cm) were selected because spoilage related to atmospheric exposure usually occurs at near-surface locations. The sensors were programmed to monitor the temperature and oxygen concentration inside the silage stack and transmit data to a gate-way placed on top of the silage stack, directly above the sensors. The maximum distance between sensors and gateway was approximately 1 meter. The stack was covered with two layers of black 0.15 mm poly-ethylene. The experiment was carried out over a period of 102 days, starting 6th October 2006. Climatic data (air temperature and soil temperatures at depths of 10 and 30 cm) were collected by the Danish Meteorological Institute, Copenhagen, Denmark from the nearest national climate station (6174, Køge/Herfølge), located approximately 10 km from the experimental farm,.

Results and discussion

Hand-held gas detector

The results of the monitoring employing method A are presented in Figure 3.4. The oxygen levels in both the sampled stacks were between 0 and 1% throughout the study. The only exception is the last visit at FK where a field stack was sampled as the silo stack was used up. Here O_2 concentrations were on average 1.6%. Also a single measurement of 3.8% O_2 was obtained at FK in July. This may have been caused by intruding atmospheric air. The standard deviations between measurements were in the range 0.1-0.6 for O_2 and 0.6-2.6 for CO_2, when omitting the high O_2 measurement at FK in July as an outlier. It is thus possible to measure the general levels of O_2 and CO_2 in a silage stack, but the risk of intruding air during measurement makes is difficult to give accurate estimates. Particularly for O_2 where the difference between <0.5 and 1% can be decisive for growth of micro-aerophilic fungal species (Taniwaki et al., 2001).

Gas measurements conducted in 20 maize silage stacks in connection with the January sampling in **Paper II** ranged between 0.2 and 9.3% O_2 and 10.3 to 34.5% CO_2 (Appendix A). These measurements were taken in sample holes deeper than 1 m, increasing the risk of intruding air considerably. The measurement at farm 7 with 9.3% O_2 and 10.3% CO_2 illustrates this. Reference measurements in this stack conducted by inserting the probe 20-30 cm from the cutting front showed 1-2% O_2 and approximately 18% CO_2 but it is not possible to insert the probe further and the pump has difficulty pumping sufficient air.

All the CO_2 measurements are low compared to the concentrations reported by Williams et al. (1997) and Weinberg and Ashbell (1994), while O_2 measurements were comparable. There are various possible explanations for this. The concentrations may simply have been lower due to a relatively short distance to the surface. Ashbell and Weinberg (1992) detected CO_2 levels between 20 and 40% in the top layers of maize silage. Mixing with atmospheric air during sampling could also have caused the discrepancy, but that should also have raised O_2 levels. The CO_2 sensor was only designed and tested for 0-25% CO_2, so higher levels may not have been detected correctly. No cross-

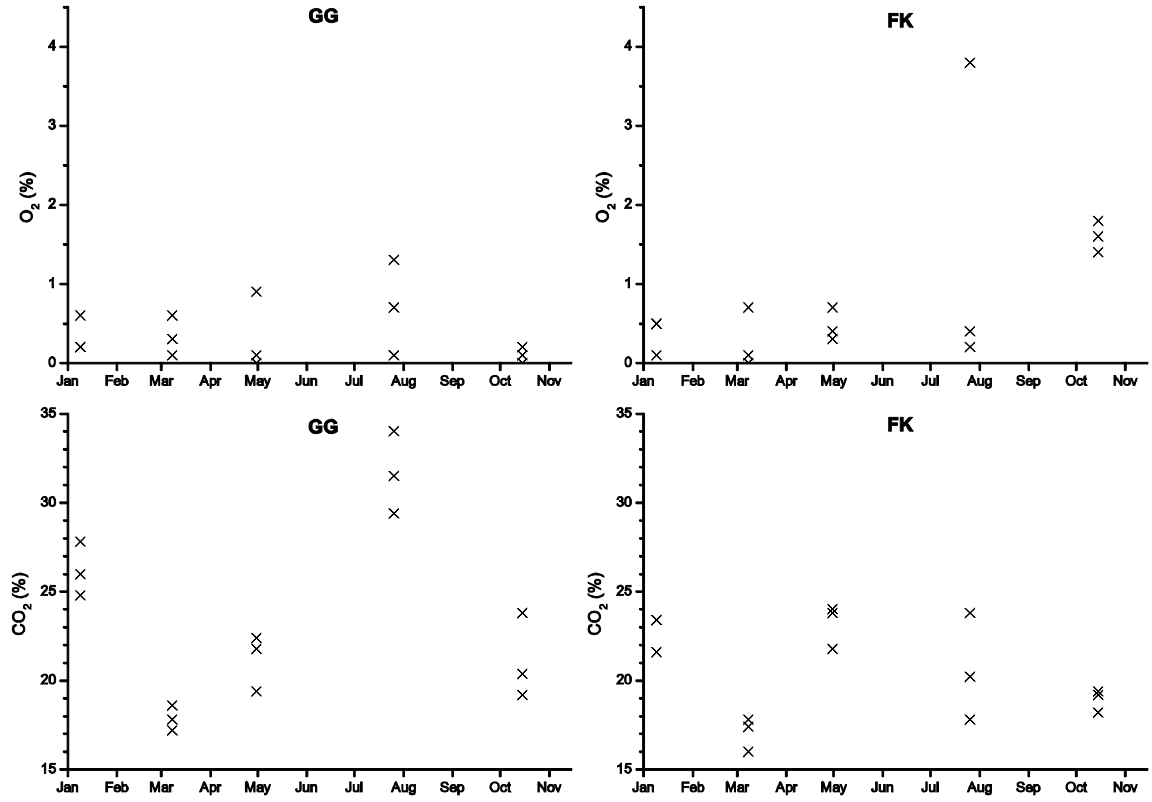

Figure 3.4: Results of the measurements of oxygen (upper graphs) and carbon dioxide (lower graphs) with the handheld gas detector at Gjorslev Gods (GG) and Freeslev Kotel (FK) during 2007.

sensitivity was reported for the CO_2-sensor by the producer, but the special conditions in silage may have an effect. The O_2 sensor was sensitive to prolonged exposure to high levels of CO_2. According to the detector manual this would increase the O_2 signal by approximately 0.3% for each % CO_2. Therefore the sensors were flushed with atmospheric air between each measurement.

Wireless sensors

The temperatures detected by the sensors are displayed in Figure 3.5 together with average daily air temperatures. The silage temperatures are generally above air temperatures, and temperature at a depth of 50 cm is higher than at 25 cm. It is also evident that the temperature 25 cm in the stack is dependent on air temperature and fluctuates with it. At 50 cm depth the temperature is much more stable. After the initial increase due to microbial activity during fermentation the temperature gradually decreased to around 11.6^{o}C in January. The temperature sensors continued to function for at least 102 days. Unfortunately problems were encountered with the transceiver in the gateway. It turned of twice, possibly due to power-cuts, and had to be manually restarted. Consequently some values are missing in Figure 3.5.

The oxygen sensor in sensor unit B25 only lasted a very short time. The atmosphere of the silage very quickly destroyed the electronics of the sensor, because these were not sealed in this prototype of the instrument. Therefore no oxygen data were recorded. Later models of the sensor remained operative for up to 1 month (Ole Green, personal communication).

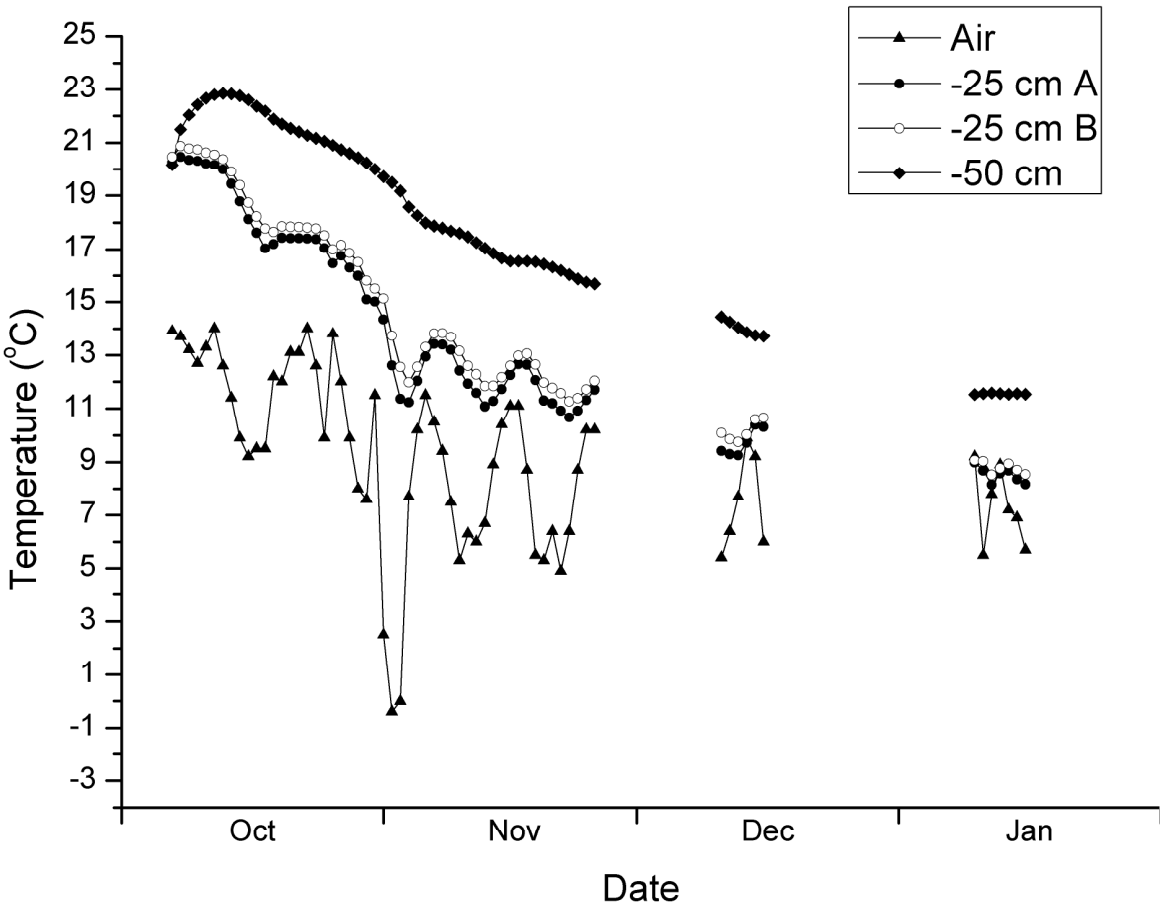

Figure 3.5: Average daily temperatures recorded by wireless sensors 25 and 50 cm from the surface of the silage stack together with average daily air temperatures.

Conclusion

The hand-held gas detector was capable of making approximate measurements of O_2 and CO_2 *in situ*. O_2 concentrations were generally below 1-2% and CO_2 concentrations were between 15% to >25%. Further improvements, adaptations and testing of the procedure is however necessary to ascertain the accuracy of the measurements.

The prototype wireless sensors were capable of monitoring and transmitting silage temperature continuously for 53 days and the sensors were functional for at least 102 days, disregarding the problems with the transceiver. An improved transceiver, e.g. with some data storage capability would alleviate this problem. The results show marked day-to-day variations in the temperature 25 cm into the stack, following ambient air temperatures, while only very weak day-to-day variations were detectable at a depth of 50 cm.

3.4 Development of an LC-MS/MS method for the detection of 27 mycotoxins and other fungal metabolites in maize silage

Ida ML Drejer Storm and Rie R Rasmussen

A paper covering this work is in preparation

Introduction

Maize silages can be contaminated with a wide variety of fungal metabolites originating from both pre-harvest and post-harvest contamination (**Paper I**). To enable fast and reliable estimation of the total intake of mycotoxins through silage there is a need for non-selective multi-methods for the simultaneous extraction and detection of many diverse fungal secondary metabolites in silage.

In the last few years multi-mycotoxin methods have been developed and validated for various food and feed matrices. Reviews on the subject are covered by Zöllner and Mayer-Helm (2006) and Krska et al. (2008). The method of choice in these applications is liquid chromatography coupled with single or tandem mass spectrometry. Due to the high selectivity of the MS-detector(s) more matrix compounds can be tolerated in the extracts and therefore less selective extraction methods can be used. Many of the methods mentioned in the above reviews are focusing on the toxins covered by regulations, e.g. aflatoxins B1, B2, G1, G2, M1, ochratoxin A, patulin, deoxynivalenol (DON), zearalenone, fumonisin B1 and B2, T-2 and HT-2 toxin included in the European Commission regulation no. 1881 (EC Commission, 2006b). They therefore focus on analysing food for human consumption, often cereal-based products.

A few studies have also been published where multi-mycotoxin methods have been applied and tested on silage. The method by Driehuis et al. (2008) covers 20 analytes but only four are post-harvest contaminants and none are associated with *Aspergillus fumigatus*. The method by Garon et al. (2006) detects seven mycotoxins of which three are post-harvest but none are from the very common silage fungi *Pen. roqueforti* and *Pen. paneum*. Two other studies (Mansfield et al., 2008; O'Brien et al., 2006) focus on the metabolites from these two fungi but do not include any other. Ideally a method for the screening of maize silage samples should include fungal metabolites from all the primary fungi in maize silage as well as known potent mycotoxins, even though the risk of their presence is small.

Some chemical properties of silage must also be considered in the development of an analytical method for this matrix. Maize silage is a varied matrix due to the use of the whole maize plant. e.g. it contains chlorophyls and carotenoids from the leafy parts of the plant (Hopkins, 1999), starch from the cob and organic acids from the fermentation which may affect the extraction and analysis of fungal metabolites. In particularly pH may vary, from 3.6 in well ensiled maize to 7-9 in hotspots of fungal growth (Müller and Amend, 1997). This may greatly affect the chemical properties of the analytes, as illustrated in Figure 3.6. Going from pH 4 to 7, some compounds become less polar, some become more polar and some are unchanged. Such changes in polarity can affect the extraction efficiency and chromatographic properties of the compounds.

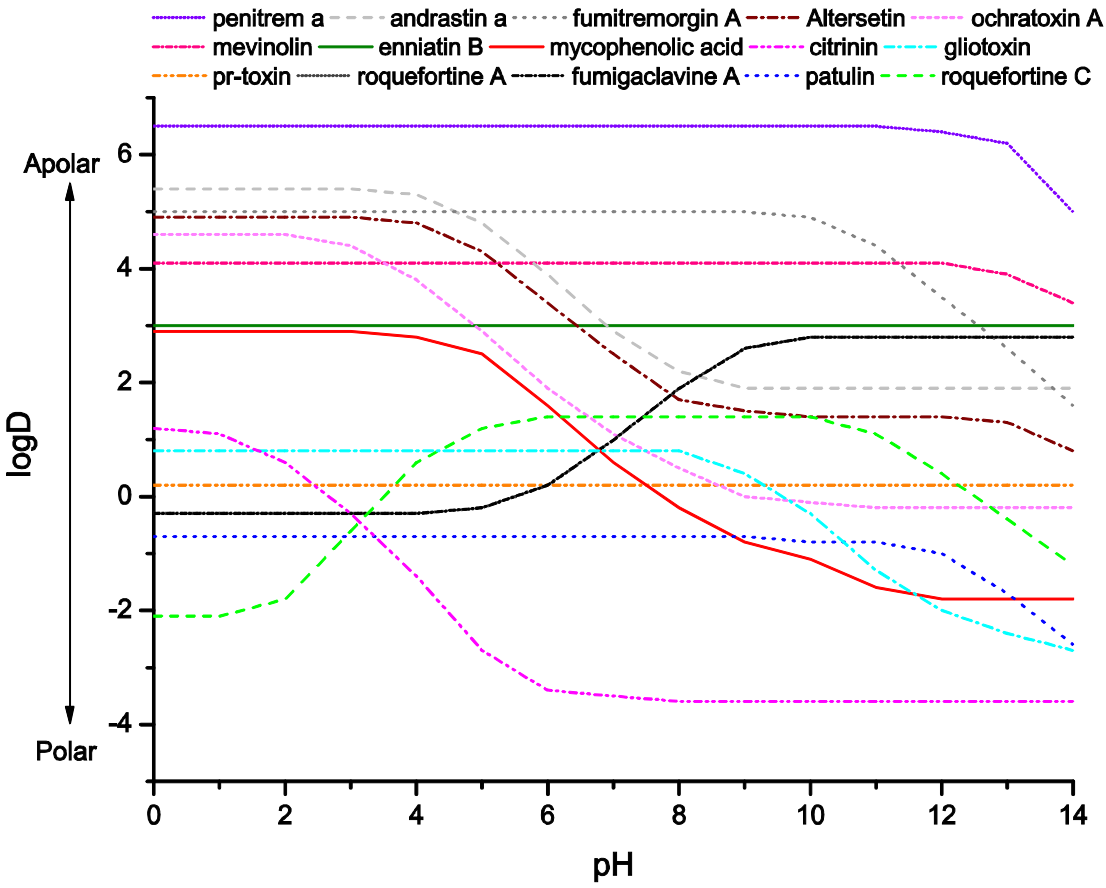

Figure 3.6: Calculated LogD values for selected fungal secondary metabolites (ACD/Labs, 2008). They estimate the polarity of the metabolites at different pH values.

Trace analysis of pesticide residues in fruit, vegetables and cereals is in many ways comparable to mycotoxin analysis. An increasingly popular multi-method for pesticide detection in various matrixes in the Quick, Easy, Cheap, Effective, Rugged and Safe method, known as QuEChERS (Anastassiades et al., 2003; Lehotay et al., 2005a; Lehotay, 2007). As indicated by the name the method is very simple and fast compared to traditional extraction and solid phase extraction (SPE) clean-up. Validation has also proven it to be stabile in spite of changes in the matrix. A buffered version of the method exists (Lehotay et al., 2005b), which also minimises the effect of changes in matrix pH.

The aim of the present study is to adapt, apply and evaluate the QuEChERS method to the extraction of 32 pre- and post-harvest mycotoxins and other fungal secondary metabolites in maize silage samples and to develop an LC-MS/MS method for the detection of these mycotoxins in the silage extracts. Twenty-seven analytes were successfully validated. To our knowledge it is the first application of the QuEChERS method to mycotoxin analysis.

Materials and methods

A detailed list of materials and methods employed are included in Appendix B.

Briefly, 10 g of homogenised silage was extracted with 10 ml 1% acetic acid in acetonitrile 5 ml water and 1.67 g sodium acetate trihydrate by vigorous shaking for 2 min. Addition of 4.0 g anhydrous magnesium sulphate induced a phase separation in the extract into a polar water phase with high

concentration of salts and a semi-polar acetonitrile phase. Further extraction and partitioning between the two phases was facilitated by shaking for 2 min. The phases were completely separated by centrifugation for 10 min. The upper acetonitrile phase was decanted and used directly for analysis after filtration.

Samples were analysed by reverse phase high performance liquid chromatography (RP-HPLC) followed by tandem mass spectrometry with electrospray ionization (ESI). Samples were analysed in two separate runs, one in positive ESI mode (ESI+) and one in negative ESI mode (ESI-). Parameters for the mass spectrometric detection of all analytes are included in Appendix B. the LC-MS/MS method was characterised for 32 compounds but only 30 were available for validation and 27 were successfully validated. The characterised compounds are: alternariol, alternariol monomethyl ether, altersetin, andrastin A, citreoisocoumarin, citrinin, cyclopiazonic acid, deoxynivalenol, enniatin B, fumigaclavine A, fumigaclavine B, fumigaclavine C, fumitremorgin A, fumitremorgin C, fumonisin B1, fumonisin B2, gliotoxin, marcfortine A, marcfortine B, mevinolin, mycophenolic acid, nivalenol, ochratoxin A, patulin, penitrem A, PR-toxin, roquefortine A, roquefortine C, sterigmatocystin, T-2 toxin, tenuazonic acid and zearalenone. They represent fungal species isolated from maize pre-harvest (*Fusarium culmorum, F. graminearum, F. avenaceum and Alternaria tenuissima, Aspergillus flavus*) and post-harvest contaminants from silage (*Aspergillus fumigatus, Monascus ruber, Penicillum roqueforti, P. paneum, Byssohchlamys nivea*).

The validation included 3 series performed on 3 different days by two different persons. Samples of a silage with a low background content of toxins were spiked quantitatively with a mixed mycotoxin standard at a low, medium and high level. The exact concentrations differed between toxins depending on sensitivity (Table 3.5). For compounds that were not available as quantitative standards a mixture of fungal extracts was used which contained these compounds in unknown amounts. The fungal extract was spiked at one level and recovery evaluated by comparison to a matrix matched dilution at the same theoretical concentration. Quantification of the 18 quantitatively validated analytes was done by comparison to matrix matched standard curves. All spiking and blank samples were prepared in triplicate. LOD was calculated as 3 times the standard deviation divided by the recovery of the lowest accepted spike level.

Finally, the method was applied to 4 samples of naturally contaminated maize silage.

Results and discussion

The method was capable of extracting and detecting 27 fungal secondary metabolites. Figure 3.7 and Figure 3.8 illustrate the abundance of the multiple reaction monitoring (MRM) chromatogram traces of all analytes at the lowest accepted spike level relative to the trace of a blank maize silage. Eighteen of the analytes were validated quantitatively and 9 qualitatively. The results of the validation are presented in Table 3.5. The method performance was comparable to other multi-methods validated for maize and grass silage (Driehuis et al., 2008; Garon et al., 2006; O'Brien et al., 2006) for most of the analytes.

The LC-MS/MS method can also screen for fumigaclavine B and fumitremorgin C. However these were not present in sufficient amount in the fungal extract to be validated. Although citrinin and Fumonisin B1 and B2 were included in the validation, they are not included in the final method as the validation results were unsatisfying.

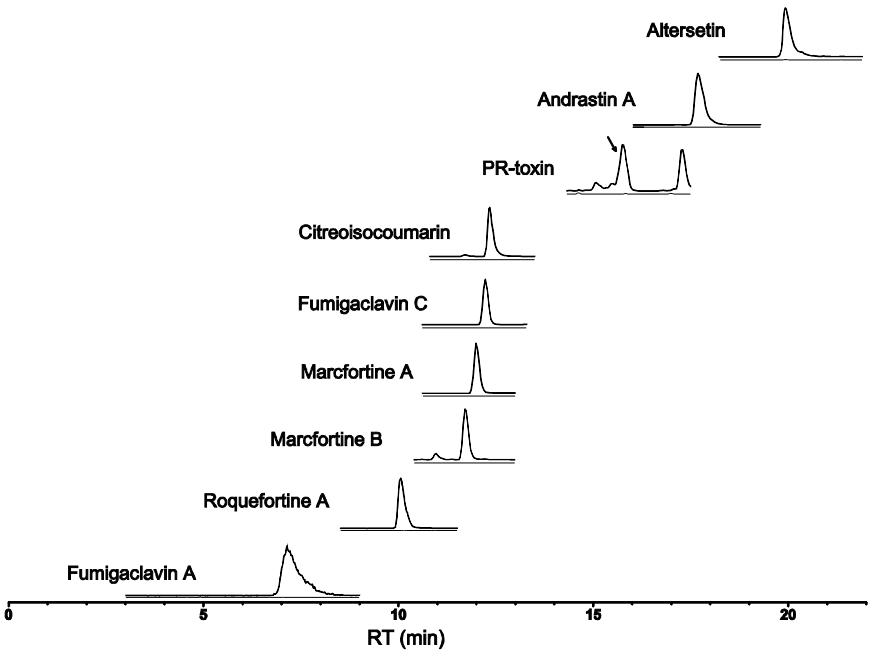

Figure 3.7: MRM traces of analytes evaluated by spiking with fungal extract relative to signal of unspiked sample

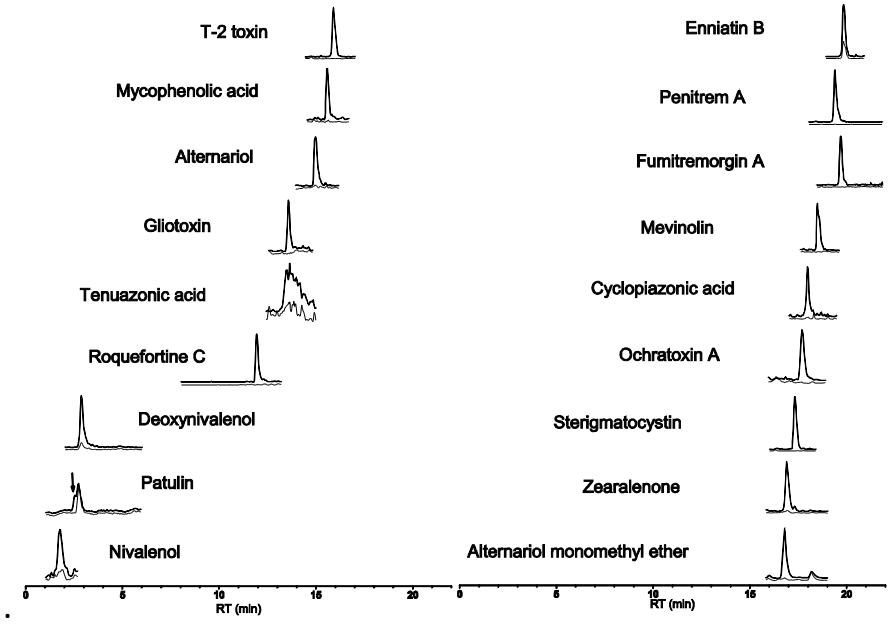

Figure 3.8: Quantitative analyte MRM traces at the lowest accepted spike level (Table 3.5) relative to trace signals in unspiked sample.

Extraction

The application of the adapted QuEChERS method to mycotoxin extraction was successful. Mean recoveries ranged from 37-201% and the majority were between 60 and 115%. The principle of the method is to combine extraction with a liquid/liquid partitioning. The extraction solution is comparable to extraction methods employed by (Driehuis et al., 2008; Garon et al., 2006; Mansfield et al., 2008; Sulyok et al., 2007). They use acetonitrile (or methanol) with 10-20 % (v/v) water, while

the present method has a 2:1 (v/v) mix of acetonitrile and water, which should allow for better extraction of the more polar analytes. With the induction of phase separation the extraction of less polar compounds is facilitated. According to Anastassiades et al. (2003) the acetonitrile phase holds approximately 8% of water. The high concentration of salt in the water phase forces the polar analytes into the less polar acetonitrile, rather than remaining in the water phase. In the case of varying water content in silage samples the phase separation should also result in a more stable polarity of the extract. This is of relevance as the microbial activity in fungal hot-spots in silage causes a much higher moisture content in this than in not infected silage.

Only fumonisin B1 and B2 of the 32 LC-MS/MS characterised compounds had unacceptably low recovery rates (6 and 13%, respectively), which is assumed to be due to poor extraction. These analytes each contain 4 carboxylic acid groups making them very polar at pH values above approximately 4-6 (ACD/Labs, 2008). Further acidification of the extraction solvent might improve their extraction but can conversely reduce the extraction of other analytes.

The buffering incorporated in the method was very effective. In a silage sample adjusted to pH>10 and subsequently subjected to the QuEChERS extraction the pH of the acetonitrile (MeCN) phase (diluted 1:4 v/v with water) was 4.3 (n=3). For the same silage at its natural pH of 4.2 the pH of the MeCN phase (diluted likewise) was 3.7 (n=3). When the same silage samples were subjected to traditional extraction with an 8:2 (v/v) mixture of MeCN and water the corresponding pH-values of the extracts (diluted 1:4 v/v with water) were 10.4 and 4.4.

LC-MS/MS method

The use of raw extracts with many matrix compounds demands a robust chromatographic method. Early in the method development unstable retention times and rapid reduction in sensitivity were observed after just 8 injections of silage extract. A post-run cleaning procedure with injections of formic acid in acetonitrile followed by methanol and finally water (Appendix B) was introduced to prevent matrix compounds from accumulating on the column. Furthermore pure acetonitrile was injected as sample for every 9 silage samples in the sequences. With these procedures the method gave reliable and stable MS/MS signals throughout a sequence.

For a few compounds the chromatographic behaviour was not optimal. Tenuazonic acid and fumigaclavine A had broad peaks, the latter also tailing (Figure 3.7 and Figure 3.8). A large matrix peak was also eluting close to the quantitative product ion of patulin (Figure 3.8), making it impossible to quantify on the basis of peak areas. Therefore height was used as response variable for patulin, which resulted in acceptable validation results. The second product ion of patulin did not suffer from matrix interference, but its sensitivity was to low for it to be useful for quantification.

The method employs both ESI+ and ESI- and the analytes detected in each mode are seen in Table B-1 (Appendix B). Mycophenolic acid, ochratoxin A and roquefortine C was detected in both modes, but ESI- was preferred due to better validation results.

Method performance

The LODs were good or acceptable for the majority of the validated analytes. The maximum recommended concentrations of zearalenon, DON and ochratoxin A in feed (EC Commission, 2006a) can easily be determined. Comparing with reported concentrations of various mycotoxins in maize

Table 3.5: Recovery, repeatability, reproducibility for the accepted spike levels. LOD was calculated from samples spiked at the lowest accepted level.

Compound	Spike levels (ug/kg)	N (spike)	Mean recovery (%)	Repeatability RSD_r (%)	Reproducibility RSD_{IR} (%)	LOD_{spike} (µg/kg)
Alternariol	20, 40, 80	27	78	9	14	10
Alternariol momomethyl ether	20, 40, 80	27	79	5	10	6
Altersetin	fungal[c]	9	91	14	14	-
Andrastin A[a]	fungal[c]	6	115	10	11	1
Citreoisocumarin	fungal[c]	9	84	7	7	-
Cyclopiazonic acid[ab]	20, 40, 80	18	63	22	35	15
Deoxynivalenol[b]	1399, 2797	18	83	17	18	739
Enniatin B	20, 40, 80	27	61	20	22	19
Fumigaclavine A	fungal[c]	9	93	12	21	-
Fumigaclavine C[ab]	fungal[c]	6	170	13	16	-
Fumitremorgin A[b]	100, 200	18	93	18	23	76
Gliotoxin	200, 400, 800	27	85	13	13	71
Marcfortine A	fungal[c]	9	63	12	16	-
Marcfortine B	fungal[c]	9	61	9	9	-
Mevinolin[b]	40, 80	18	68	25	27	25
Mycophenlic acid	20, 40, 80	27	90	11	13	7
Nivalenol[b]	200, 400, 800	27	68	13	15	122
Ochratoxin A	40, 80	18	71	8	9	10
Patulin	700, 1400, 2800	27	100	17	17	371
Penitrem A	20, 40, 80	27	107	6	12	8
PR-toxin	fungal[c]	9	56	27	32	-
Roquefortine A	fungal[c]	9	103	13	32	-
Roquefortine C[a]	fungal, 200, 400, 800	24	201	9	26	158
Sterigmatocystin	20-80	27	72	9	9	8
T-2 toxin[b]	125, 250, 500	27	55	17	26	96
Tenuazonic acid[b]	fungal, 202, 404	27	37	20	20	121
Zearalenone	20, 40, 80	27	90	12	16	9

[a]Data from day 2 omitted due to high day-to-day variation in recovery.
[b]ion ratio out of the expected range for a significant number of samples.
[c]Samples spiked with mixed fungal extract containing the analytes in unknown concentrations. Validation results are only valid for this unknown concentration level.

silage samples (Driehuis et al., 2008; Mansfield et al., 2008; Sørensen et al., 2008), the majority of mycotoxin contaminated samples would have been detected with the present method. Only for DON, patulin and roquefortine C the LODs were high compared to other methods. DON and patulin were difficult to quantify consistently in this method due to high interference of polar matrix compounds and the high LODs were considered acceptable for the research purposes of the method. Other methods exist if high accuracy and precision for their determination is necessary.

Roquefortine C had both a high LOD, the highest recovery rate and a relatively high reproducibility. An unacceptable day-to-day variation was also seen on day 2 of the validation for this analyte together with andrastin A, cyclopiazonic acid and fumigaclavine C. This indicates that the robustness of the method for particularly these compounds should be explored further. Incorporation of internal standards in the method should improve both LODs, repeatabilities and reproducibilities.

The method was applied to 4 naturally contaminated silage samples (Table 3.6). Reported are compounds, which were above the LOD and met the identification criteria. Three of the samples were visibly moldy hot spots of maize silage. The last sample was a mix of silage from stacks and *Penicillium* hot spots selected from stacks. This was included as a 'control' sample during the validation.

Table 3.6: Fungi and fungal secondary metabolites detected in 4 samples of maize silage with visible fungal growth. Concentrations are reported with 95% confidence intervals calculated according to the validation results.

Sampling	hot spot	hot spot	hot spot	hot spot + stack
Isolated fungi	*B. nivea* *M. ruber*	*Pen. paneum* *Pen. roqueforti*	*Pen. paneum* *Pen. roqueforti*	*Pen. paneum* *Pen. roqueforti*
Compound (ug/kg)*				
Andrastin A		4,895 ± 1235	8,811 ± 2223	18 ± 5
Deoxynivalenol				1092 ± 454
Enniatin B	37±19			
Mycophenolic acid		335 ± 96	407 ± 117	
Nivalenol			138 ± 43	142 ± 44
Roquefortine C		1,765 ± 948	33,662 ± 18,082	51 ± 27
Zearalenone				9 ± 2
Qualitative				
Citreoisocoumarin		X	X	X
Marcfortine A		X		
Marcfortine B		X		
Roquefortine A		X	X	

Conclusion

A new method for detection of 27 fungal secondary metabolites in maize silage was developed and successfully validated. Eighteen of the analytes can be detected quantitatively and 9 qualitatively with recoveries from 37 to 201%, LODs from 1 to 739 $\mu g \cdot kg^{-1}$ and reproducibilities from 7 to 35%. A pH buffered extraction method ensured the same extraction conditions for fungal hot spots (pH 6-7) and normal silage (pH 3-4). Applied to 4 Danish maize silage samples the following analytes were identified: andrastin A, citreoisocoumarin, marcfortine A and B, mycophenolic acid and roquefortine A and C (*Pen. paneum* and *Pen. roqueforti* metabolites) together with the pre-harvest mycotoxins enniatin B, nivalenol, zearalenone, and deoxynivalenol (*F. graminearum* and *F. avenaceum* metabolites). The highest detected concentration was 34 ± 18 $mg \cdot kg^{-1}$ of roquefortine C.

4 Discussion

Trying to understand the complex microbial ecosystem of a silage stack is not an easy task. The basic principles are well understood, but the diversity of nature constantly presents us with exceptions to these principles. To overcome these unavoidable sources of variation and only deal directly with the principles, many silage studies employ laboratory scale experiments. In the laboratory, conditions can be controlled, eliminating many of the variables in play in full-size silage stacks. This makes it easier to reach conclusions, but they may not hold in the more complex ecological system in the field.

The purpose of this PhD study is to gain knowledge about fungal spoilage of maize silage in Denmark. Therefore, focus has been on monitoring and documenting what happens in the field. All the conducted work is related to documentation of the situation in normal full-size silage stacks or methods for this. This means that the observations pertain to Danish conditions and may differ with other agricultural practices, climates etc. As the studies only include silages from 2005-2007, with the majority from 2006, year-to-year variations are not fully covered either.

A general problem in science is that any study of an object or system can have an effect on the object or system itself. This is very pronounced in the study of silage, as sampling of silage stacks has the potential to affect atmospheric composition and thereby the microbial composition of both sample and the remaining silage. The possible effects of sampling and sample handling methods must therefore be considered carefully in the interpretation of results.

A consequence of the focus on full-size stacks is that sampling becomes a very important issue. As documented in **Paper III** fungi can be very heterogeneously distributed in maize silage stacks. It is therefore necessary to collect and analyse many samples in order to draw conclusions about a whole stack or about silage stacks in general. Limited studies of one or a few silage stacks make little sense in themselves, but reviewed with other similar studies, patterns can emerge. An example is the use of *L. buchneri* as silage additive, which has been tested by many scientists on different crops with varying results. A meta-analysis of 43 experiments in 23 studies revealed significant effect of *L. buchneri* as additive in maize silage on pH, lactic and acetic acid concentrations, yeast counts and aerobic stability (Kleinschmit and Kung, Jr., 2006). Effect of *L. buchneri* was also seen for grass and small-grain silages, but the difference in aerobic stability between treatments was much more moderate. Limited studies, as **Paper III** should therefore also be seen as contributions to the combined knowledge of the scientific community.

4.1 Fungi

The monitoring described in **Paper II** clearly documents that viable fungal propagules are ubiquitously present in Danish maize silages. Filamentous fungi were isolated from all the collected samples from hot-spots and/or visibly non-mouldy samples. All the sampled silages therefore had the potential to develop hot-spots of fungal growth. Considering the many other reports of filamentous fungi in maize silages from around the world (see Chapter 1.4 and **Paper I**) it is likely that this potential for fungal spoilage is general and unavoidable.

The identified species of filamentous fungi in **Paper II** are in accordance with the review of publications on the subject in **Paper I**. *Pen. roqueforti* and *Pen. paneum* were the dominant species

and either or both were isolated from 96% of the collected samples. The two species can be very difficult to separate in culture as they are very similar and both sporulate profoundly. In personal experience *Pen. roqueforti* spores are slightly more hydrophobic and spread more easily, so the presence of *Pen. paneum* may have been overlooked in previous reports. The distinction between *Pen. roqueforti, Pen. paneum* and *Pen. carneum* was only introduced in 1996 (Boysen et al.) so studies pre-dating this time contain no reports of *Pen. paneum,* either. The third member of the *P. roqueforti* complex (Boysen et al., 1996), *P. carneum*, was not observed, which is in accordance with (Boysen et al., 2000).

The *Zygomycetes* were also very common with *Mucor* spp. as the most frequent. This is also in accordance with **Paper I** and Pelhate (1977). The isolates were not identified to species level so it is not known whether only one or several different species were common. They often co-occurred with other species during isolation, and due to their rapid growth they often dominated the other fungi, especially on V8. In many of these cases it was impossible to isolate the other speices and only tentative identifications of them were possible.

A. fumigatus is often emphasized in mycological surveys of silage due to its production of many bioactive secondary metabolites (Frisvad et al., 2009) and the risk of infections in lungs and other tissue (Aspergillosis) (Jensen et al., 1994; Latge, 1999). The percentage of stacks infected with *A. fumigatus* in the present survey ranged from 11-60 %, peaking in May after 7-8 months of ensiling. In literature, the incidence of *A. fumigatus* in silage is very variable ranging from 8-9 % of samples (Nout et al., 1993; Schneweis et al., 2001) to 69-75 % of samples (dos Santos et al., 2002; El-Shanawany et al., 2005). This places the data of the present survey in the same range, indicating that the differences may be due to different times of sampling or possibly climatic variations. *A. fumigatus* colonies on the initial plates of V8 and DG18 were neither dense nor easily distinguishable and they often co-occurred with other species requiring experience to spot and separate them.

M. ruber was on average not commonly detected in **Paper II**, but were described as very common by Schneweiss et al. (Schneweis et al., 2001) with a frequency of 16 and 20% in visibly mouldy samples of maize and grass silage, respectively. Others report much lower frequencies of 1.5% for *Monascus* spp. (Gedek *et al.*, 1981) cf. (Nout et al., 1993) and 4% in silage of sugarbeet press pulp (Nout et al., 1993). These differences could, as for *A. fumigatus*, be related to sampling time. In **Paper II** *M. ruber* was primarily present in the January samples with a frequency of 25% of the stacks, while very few isolates were collected later in the season. Pelhate (Pelhate, 1977) also describes this species as one of little competitive power which is often displaced by other fungi.

B. nivea/*Pae. niveus* occurred quite consistently in **Paper II** with frequencies of 10-25%. Much higher frequencies were observed by Escoula (1974) and Hacking and Rosser (1981) when they were specifically searching for *B. nivea* and *Pae. niveus*, respectively. In a survey of big bales of grass silage (Skaar, 1996) only 1-5% of the samples contained *B. nivea*. This illustrates that the purpose and design of the experiment may have an influence on the results, in particularly when subjective evaluations are involved.

Only very few Fusaria were isolated in the present study. Fusaria are however widespread in maize pre-harvest (Placinta et al., 1999) and therefore *Fusarium* mycotoxins may be present after ensiling, which was also the case in the January samples in **Paper II**.

The use of classical microbial methods of cultivation and identification on Petri dishes has both advantages and disadvantages. It is a long established and well documented method and the morphology of fungi on Petri dishes is the traditional identification criteria for different species. It also yields fungal isolates for *in vitro* examinations. Unfortunately this approach may not reflect the actual growth and abundance of microorganisms in the silage *in situ*. The specific growth conditions of the isolation procedure also affect the results, which was already mentioned in the review of silage mycobiota by Pelhate (1977). Enumeration of filamentous fungi is also highly dependent on degree of sporulation and other species specific properties. Molecular biological methods are in theory able to isolate DNA from all the species present in the silage, thus avoiding the selective culturing conditions. Reports on the application of DNA-based methods to determine bacterial profiles of silages are now common (Brusetti et al., 2006; Dellaglio and Torriani, 1986; McEniry et al., 2008a; Naoki and Yuji, 2008; Rossi and Dellaglio, 2007; Schmidt et al., 2008; Stevenson et al., 2006). The application of DNA-based techniques to fungi in silage samples is reported by May et al. (May *et al.*, 2001) and Mansfield and Kuldau (2007). The latter detected a higher number of species with the molecular technique than with traditional selective plating. Richard et al. (Richard *et al.*, 2009) also employs a PCR method but uses it to identify the fungal species present in mixed cultures of fungi isolated from silage. The molecular biological methods do however also involve selective screening and identification steps and may thus not give a true picture of the species represented in silage either. With the rapidly increasing knowledge on fungal DNA sequences, new primers could be developed both for general and selective purposes.

4.2 Growth conditions

Viable propagules of filamentous fungi are generally present in all maize silage stacks (**Paper II**), but growth of filamentous fungi is only seen in some stacks while other stacks can remain free of fungi for extended periods of time. The growth and proliferation of the fungi in the silage must therefore be controlled by external and internal factors. Throughout the ensiling and storage the silage forms a complex microbial ecosystem. The ideal system is anaerobic and dominated by LAB which can remain stable for months and years, but small deviations can displace the balance and lead to fungal growth. The highly heterogenous distribution of fungal propagules described in **Paper III** indicates that these external factors are also heterogeneously distributed.

Chemical and microbial composition

No connections between the chemical and microbial composition of silage (chemical parameters vs. the logCFUs of LAB, yeasts and filamentous fungi) were evident in **Paper II**. The chemical parameters where also much more homogenously distributed than the fungal parameters in **Paper III**. Acetic acid is proven to increase aerobic stability of maize silage and impair fungal growth (Danner et al., 2003). A negative correlation (R^2=0.66) between logCFU of yeast and acetic acid concentration in maize silages was observed by Kleinschmit and Kung (2006) in their meta-analysis of inoculation studies. No effect of acetic acid was however seen in **Paper II**, which could be due to the relatively low concentrations. The concentration of acetic acid ranged from 6.8-29.3 g·kg DM^{-1} in **Paper II**, which is reasonably close to the country averages reported in Table 1.1, while the studies by Danner (Danner et al., 2003) and Kleinschmit and Kung (2006) have concentrations from 22 up to 60-80 g·kg DM^{-1}.

A connection between logCFU of filamentous fungi and logCFU of yeasts could also be expected on the basis of the effects of yeast on aerobic degradation (McDonald *et al.*, 1991; Pahlow *et al.*, 2003). However, the correlation coefficients between yeast and filamentous fungi were below 0.2 in **Paper II** indicating no apparent connection. In **Paper III** the correlations were 0.7 and 0.8 but the very limited amount of filamentous fungal counts limits the applicability of that result. The relatively long delay between sampling and microbial analysis may have obscured the connection between microbial parameters. The average logCFU of yeasts was 5.9 in **Paper II** which is higher than the concentrations found in the untreated samples in (2006). Middelhoven (1998) and McDonald et al. (1991) mention 10^5 yeasts per gram silage as a high number of yeast. Examination of the species composition of yeast and LAB populations during the studies could perhaps reveal some connections.

Aeration

Another factor which may very well have affected the occurrence of filamentous fungi is aeration of the silages. In a study comparing effects of various preparation methods, including air infiltration, on the chemical and microbial composition of lab-scale grass silages, air infiltration was found to have a highly significant effect on the microbial composition and loss of DM (McEniry *et al.*, 2007). O'Brien et al. (O'Brien *et al.*, 2007) found visible damage to the wrapping of baled grass silage to be the only production and storage factor that significantly increased fungal spoilage.

Most of the hot-spots described in **Paper II** were found near the surface or sides of the stacks. They were present either as lumps from 5 to 20-30 cm in diameter or as areas with many small hot-spots on or near the surface. Sampling of surface hot-spots with limited extent was avoided. Occasionally single lumps or areas with several lumps were observed in the centre of stacks. The position of hot-spots was noted on a stack diagram, but no objective measure of the distance to the surface was recorded, so it is difficult to classify the hot-spots as "surface" or "interior" consistently. An analysis of this aspect of the results could have been interesting.

Growth on the surface is likely the result of insufficient silage management. If the silage cover is not kept to the surface of the stack constantly, intruding air will allow growth of various microorganisms (Chapter 1.3). Occasionally the upper layers of silage stacks in **Paper II** were in advanced stages of aerobic degradation with recorded temperatures as high as 65^{o}C. The occurrence of hot-spots or layers of grey-green or blue-green fungi near but not on the surface was also observed by Auerbach et al. (1998), Nout et al. (1993) and Ida Storm (Personal observation, Figure 1.5). This could be the result of fungal specialisation in the microbial ecosystem. Due to loose silage covers and less compaction of the upper layers, oxygen is able to diffuse a limited distance into the stack. In the outer most layers yeasts and/or bacteria are more competitive than the filamentous fungi, but further towards the centre the microaerophilic fungi have a competitive advantage and dominate.

Several factors may affect the aeration of silage when the cutting face is exposed to atmospheric air. Connections between silage management practices and silage preservation was examined by Ruppel et al. (Ruppel et al., 1995) who found that packing intensity during silo packing was associated with increased DM density, lower DM loss and higher aerobic stability. No significant effect of feed-out rate was registered. Models describing the permeability of silage to air also operate with the parameters density, DM and porosity (Pitt and Muck, 1993; Williams, 1994). It is easily perceived

that density and porosity is affected by packing intensity and they are also connected to particle size of the chopped crop (Muck *et al.*, 2003). The size and stiffness of the particles affects the ability of the silage to compact (Muck *et al.*, 2003). Effects of the equipment for removal of silage from the stack have also been documented in some studies (Muck *et al.*, 2003). The measurement of density and porosity in **Paper II** could perhaps have revealed some connections to fungal occurrences.

A significant variation in yeast counts between silos and field stacks in **Paper II** was observed at the two last samplings in July and September: yeast counts were higher in field stacks. This could be because of the higher surface-to-volume ratio in a field stack compared to a silo, particularly in light of the long storage times these stacks were subjected to prior to sampling. CO_2 concentrations in bales of grass silage with a very large surface-to-volume ratio were shown to decrease from 90 to 15% over a period of 9 months (Forristal *et al.*, 1999) while they remained high (80-90% v/v) in some maize silage silos for 4-5 months (Weinberg and Ashbell, 1994). The number of stacks of each kind in **Paper II** was limited making it difficult to make general conclusions. Further exploration of differences between stack types, e.g. with *in situ* measurements of gas composition employing the methods described in chapter 3.3, might reveal results with importance for practical silage management.

Seasonal variations

The analysis of the seasonal variations in microbial counts (**Paper II**) revealed significant differences over the course of a storage season. Initially the counts of filamentous fungi increases but after 5-7 months of storage the amount of viable fungal propagules decreased. This may be due to a reduction in the total number of fungal propagules or due to reduced viability of the propagules after more than 7 months in the silage. 3 months of airtight storage has been found to decrease the germinability of *Pen. roqueforti* spores (Richard-Molard et al., 1980). Middelhoven and van Baalen (1988) also found anaerobic silage to be a hostile environment to yeast as the yeast counts peaked during the first 14 days and then gradually decreased over a period of 4 months. A lower number of yeast and moulds should reduce the risk of spoilage upon aeration, as yeasts are believed to be the microorganisms who initiate aerobic spoilage (Lindgren et al., 1985a; McDonald et al., 1991; Middelhoven, 1998). The environment in the silage stack not only protects the silage when it prevails but also reduces the risk of spoilage upon aeration.

The number of collected hot-spots was also lower in September in **Paper II** which could be connected to the lower number of viable fungal propagules. If the hot-spots only develop as the bunker face approaches and atmospheric air diffuses into the stack, a lower number of fungal CFUs in the old silage will lead to fewer hot-spots. The rate of use and stack management has also previously been related to fungal spoilage, aerobic stability and loss of dry matter (Nout et al., 1993; Pitt and Muck, 1993; Ruppel et al., 1995).

Temperature

The possibility of seasonal temperature variations being the cause of the microbial variations instead of storage time was also examined. There was no correlation between temperatures measured 15 cm behind the bunker face at sampling (Raun, B. M. L. and Kristensen, N. B.) and any of the microbial parameters. There was significant difference in silage temperature between sampling times (Raun, B. M. L. and Kristensen, N. B.). Silage temperatures were lowest in January and highest in July,

following the same trend as air temperatures (Figure 2). Silage temperatures were higher than air temperatures, especially during winter, which can be explained by the microbial activity of the silage. As shown in **Paper IV** the temperature at depths of 25 and 50 cm in silage stacks also follow the trends of the air temperature so no correlation between temperature and microbial numbers further inside the stacks is expected.

The temperature can also affect the fungal species composition of the silage. The abundant detection of *Mucor* spp. compared to other *Zygomycetes* can be related to temperature levels as *Mucor* is the genus within the *Zygomycetes* with the lowest temperature for optimal growth (Samson et al., 2002). *Penicillium roqueforti* is capable of growing at temperatures of 5 to 10^{o}C (Frisvad and Samson, 2004; Richard et al., 2009) which may explain its abundance early in the year, where stack temperatures at the bunker faces averaged 13^{o}C. During a pilot experiment conducted in 2006 (Appendix C), abundant growth of *Pen. roqueforti* and *Pen. paneum* was observed in a maize silage stack in March, when average air temperatures for January, February and March had been -0.9, 0.6 and -0.2^{o}C (DMI, 2009), respectively. The heat resistence of *M. ruber* and *B. nivea* and high optimum temperature for *A. fumigatus* will provide an advantage to these species near aerobically degrading silage. Temperatures around 40-60^{o}C were measured near such areas of silage in **Paper II**.

4.3 Mycotoxins

On the basis of the findings of hot-spots and viable fungal propagules of mycotoxigenic fungi in Danish maize silages (**Paper II**) and other reports of post-harvest mycotoxins in silage (**Paper I** and Chapter 1.5) it is considered very likely that mycotoxins are present in Danish maize silages to some extent. In order to be able to determine the actual exposure levels and the diversity of fungal metabolites in silage a method of analysis for a variety of mycotoxins in silage was developed and applied to 4 fungal hot-spots of maize silage (Chapter 3.4 and appendix B).

Method of analysis

The overall performance of the method was satisfactory. The mean recoveries and LODs were comparable to other multi-mycotoxin methods validated for maize and grass silage (Driehuis et al., 2008; Garon et al., 2006; O'Brien et al., 2006). Some of the analytes had high values for LOD, reproducibility or recovery, but the analysis of multiple diverse mycotoxins in one method does mean that not all analytes can be extracted and detected equally well. The described method was developed for research purposes and emphasis was on the incorporation of many different fungal metabolites, representing as many of the known and theoretical silage contaminants as possible. The necessity for low limits of detection and good reproducibility was therefore not as important as for methods for food and feed control.

Extraction

The applied QuEChERS method is very simple compared to solid phase extraction. In the initial steps of the method development SPE was tested for clean-up of silage extract. Both plain reversed phase and mixed mode columns were tested without satisfactory results. In the simple reverse phase procedure only a few matrix components could be removed without removing some of the analytes. When taking advantage of the functional group on the molecules several analytes were not retained very well and the procedure was very time consuming. Even in combined extracts from the SPE clean-up large quantities of matrix were still present. In some cases, adjustment of pH in the extracts

in order to optimise SPE retention also led to phase separation of the extract, which interferes with the SPE separation. SPE clean-up therefore did not constitute an improvement.

It is possible that the extraction in the current method can be improved by performing a longer initial extraction without any salts. Lehotay et al. (2005b) did not experience any negative effect of combining the extraction and partitioning steps into one procedure. There may however be some differences between pesticides and fungal metabolites. Pesticides are applied to the outside of the product while fungal metabolites can be produced inside the plant. Optimisation of extraction time should therefore be done with naturally infected samples. Differences in the optimal extraction procedure have been observed between different matrixes for the extraction of zearalenone (Hartmann et al., 2008). The amount of sodium acetate or sodium chloride in the QuEChERS method is also shown to effect the extraction of both analytes and matrix compounds (Anastassiades et al., 2003; Lehotay et al., 2005b). Fine-tuning of this concentration could improve the balance between analytes and interferences in the extract.

The dispersive SPE with primary-secondary amin (PSA) employed in the QuEChERS methods for pesticides (Anastassiades et al., 2003; Lehotay et al., 2005b), was not used for mycotoxins. PSA binds organic acids which in our case would be mycophenolic acid, ochratoxin A and citrinin as well as tenuazonic acid and cyclopiazonic acid, which all have acidic properties. It was therefore chosen not to employ this clean-up procedure. The use of other sorbents in a dispersive SPE step is an interesting option to explore.

LC-MS/MS method

Both positive and negative ESI was employed in the developed method. The two ESI modes were employed in separate chromatographic runs, even though the MS/MS was capable of switching between modes during a run. This meant a doubling of analysis time and a marked increase in cost of each analysis. The separate runs were preferred because it made it possible to adapt the mobile phases to the ESI modes. In ESI- only a low concentration of formic acid in the watery mobile phase was applied. In ESI+ addition of ammonia and a higher concentration of formic acid ensured formation of ammonia adducts for T2-toxin and enniatin B and more consistent chromatography for the pH-dependent analytes roquefortine A, cyclopiazonic acid and citrinin. Applying the ESI+ mobile phase to ESI- runs resulted in a marked decrease in the signals particularly of the early eluting analytes.

Detected mycotoxins

The developed method of analysis has so far only been applied to 4 naturally infected maize silage samples. Further analysis of infected and visibly uncontaminated silage samples will be conducted in the near future.

In the hot spots from which *Penicillium roqueforti* and *Pen. paneum* have been isolated their associated metabolites were detected: mycophenolic acid, roquefortine A and C, andrastin A, citreoiscoumarin, marcfortine A and B. The high concentrations of mycophenolic acid, roquefortine C and andrastine A in hot spots is consistent with observation in grass silage by O'Brien et al. (2006).

Zearalenone, nivalenol, deoxynivalenol and enniatin B are toxins from *Fusarium* species infecting the maize in the field. They are ubiquitously present in Danish maize before ensiling, but usually in low

concentrations (Sørensen, 2009). They were also detected with the current method. The concentrations were near the limit of detection and much below the maximum content in feed recommended by the EC Commission (2006b).

5 Conclusions

On the basis of the conducted studies the following conclusions can be drawn

5.1 Fungi

- Filamentous fungi are ubiquitously present in Danish maize silages.
- The most common filamentous fungi in maize silage are
    - *Penicillium roqueforti*/*Penicillium paneum*
    - *Zygomycetes*, primarily *Mucor* spp.
    - *Aspergillus fumigatus*
    - with *Byssochlamys nivea*/*Paecilomyces niveus*, *Monascus ruber* and *Geotrichum candidum* occurring less frequently
- Filamentous fungi and yeasts are very heterogeneously distributed in maize silage stacks. One cannot draw conclusion about the average content of fungal propagules in a whole maize silage stack on the basis of one or a few full depth samples. The present study suggests that more than 11 samples are needed from one stack to determine an average concentration of filamentous fungi with 95% confidence limits of ± 1 logCFU.

5.2 Growth conditions

- Significant changes occur in the conditions for proliferation of filamentous fungi in maize silage over a whole storage season. Numbers of viable fungal propagules in maize silage stacks was shown to vary significantly over a storage season. Seasonal variations in the species present in Danish maize silages were also detected. Therefore the risk of fungal spoilage and mycotoxin contamination of Danish maize silage is expected to be highest five to seven months after ensilage and lowest after 11 months.
- No correlations were observed between the numbers of viable fungal propagules in maize silage samples and any of the parameters: counts of lactic acid bacteria, counts of yeasts, dry matter content, pH, temperature 15 cm behind bunker face and concentrations of ethanol, propanol, 2-butanol, propanal, ethyl acetate, propyl acetate, propylene glycol, D-glucose, L-lactate, ammonia, acetate, propionate and butyrate.
- Test of a prototype of a wireless sensor for continuous non-invasive detection of temperature inside maize silage stacks *in situ* was successful. This type of sensor may in the future be an excellent tool for documentation of temperature and oxygen fluctuations in full size silage stacks, without the bias of invasive procedures.

5.3 Mycotoxins

- Several known mycotoxigenic species of filamentous fungi are common in Danish maize silages. They can potentially contaminate silage with a wide variety of mycotoxins and other secondary fungal metabolites.
- A multi-method was developed for the extraction and LC-MS/MS analysis of 27 mycotoxins and other fungal secondary metabolites in silage samples. The method covers secondary metabolites from all the most common post-harvest fungal contaminants of maize silage, except the *Zygomycetes*. Initial validation results showed that 18 analytes could be detected quantitatively with limits of detection from 1 to 739 $\mu g \cdot kg^{-1}$ and recoveries from 37 to 201%. The majority of recoveries were between 60 and 115%. 9 analytes were determined

qualitatively with semi-quantitative estimates of recoveries. 2 analytes were detected qualitatively.

- The post-harvest fungal metabolits andrastin A, citreoisocoumarin, marcfortine A and B, mycophenolic acid and roquefortine A and C were detected in 4 naturally infected maize silage samples together with the pre-harvest mycotoxins deoxynivalenol, enniatin B, nivalenol and zearalenone. The highest detected concentration was 34 ± 18 $mg \cdot kg^{-1}$ of roquefortine C.
- A major problem for multi-mycotoxin analysis in maize silage samples was shown to be the robustness. Co-extracted matrix compounds from the silage accumulated over the course of repeated analysis resulting in reduced signals. The problem was solved with an extended cleaning procedure between analytical runs. Further tests of robustness and possible improvements should be conducted.

6 Perspectives

On the basis of the present results it is not possible to ascertain whether post-harvest mycotoxins in maize silage can cause some of the observed incidents of illness and ill-thrift in dairy cattle. The potential for fungal growth is generally present in all silage stacks and hot-spots of limited extent are often seen. However, filamentous fungi often grow in the outer layers of silage stacks so if normal sense is applied and visibly mouldy silage is discarded, the amount of contaminated silage in the feed should be limited.

In Denmark silage is generally mixed with other feed components to a total mixed ration employing large-scale machinery. Therefore fungal hot-spots in the silage will in most cases be distributed in a larger volume of feed and possible mycotoxin concentrations thereby diluted. If the hot-spots are not mixed with the remaining silage, a high intake of mycotoxins by a few cows can occur. Cows are however known to sort the feed according to palatability. On a farm visited in 2006 hot-spots had been found among leftover feed in the stable, but this does not exclude that some hot-spots were eaten.

It is also possible that poor silage management is associated with poor cow management. Through the field trips to various dairy farms during the entire study it is clear that farmers have very different approaches to the management and care of animals and the farm in general. It can therefore be difficult to distinguish whether the symptoms observed at a farm are caused by mycotoxins in the feed or e.g. insufficient bedding in the stable, to little space for each cow, inadequate hoof care or a combination of factors.

Very little is currently known about the toxicity of silage post-harvest mycotoxins to ruminants. This topic calls for further research. Synergistic effects between both pre-and post-harvest mycotoxins may exist and cause problems both in the rumen and other parts of dairy cows (Fink-Gremmels, 2008b). The possibility of immunomodulation in cows by mycotoxins is also debated and needs further exploration (Fink-Gremmels, 2008a; Oswald et al., 2005). In humans, individuals suffering from ketoacidosis, i.e. increased levels of ketone compounds in the blood in connection with for example diabetes, are predisposed for zygomycosis (Chayakulkeeree *et al.*, 2006). The ketoacidosis is shown to disturb the normal immunological response to zygomycosis (Chayakulkeeree *et al.*, 2006). If this is also the case in dairy cows they have increased susceptibility to zygomycosis and possibly also aspergillosis in the first weeks after calving. In this transition period from pregnant and non-lactating to high-yielding dairy cow, they have a negative energy balance and therefore a high risk of developing a ketosis (Baird, 1982).

The application of the developed multi-mycotoxin method to more silage samples and fungal hot-spots is a practical perspective of the conducted work. It can be used to determine general exposure levels in maize silage for cattle feed and also to explore the connection between fungal growth in maize silage and mycotoxin production, both *in vitro* and *in situ*. The production of fungal secondary metabolites is generally dependent on growth conditions and differentiation of the fungal culture e.g. sporulation (Frisvad et al., 2008). In personal experience from bales of whole-crop barley silage fungal colonies often showed no sporulation upon opening of the bales but spores emerged after a couple of days of aerobic exposure. Metabolic profiles *in situ* may therefore vary from laboratory samples.

The developed method could also be tested on other matrixes e.g. rumen fluid, blood and milk. Samples of these matrixes from dairy cows fed mycotoxin contaminated silages are available from a feeding experiment conducted at Department of Animal Health and Bioscience, Faculty of Agricultural Sciences, Aarhus University. Modifications of the method are most likely necessary, but with proper validation it would allow the estimation of both animal and human exposure and might give interesting information on the metabolism and bioavailability of mycotoxins in dairy cows.

The intrusion of atmospheric air into silage stacks seems a likely cause of many cases of fungal growth. It would therefore be interesting to use newer models of the tested wireless sensor with O_2 detectors to examine the relationship between different methods of silage management and air ingress in the stacks. If a CO_2 detector could be incorporated in the sensor even more information about changes in silage air composition over time would be available. Intruding air may not reach the sensors inside silage stacks as the microorganisms quickly use available O_2.

The results suggest that the extent of fungal spoilage of well fermented maize silage can be limited by keeping stacks well sealed for more than seven months before opening. The viability and proliferation of different fungal spores during long-term storage under silage conditions could be examined in laboratory experiments.

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Appendix A-D

Appendix A: O_2 and CO_2 measurements with hand-held gas detector

Methods and materials

The G750 Polytector II (Gesellschaft Für Gerätebau mbH, Dortmund, Germany) was equipped with a pump (0.7 l·min^{-1}) making it possible to pump air from a specific confined space through a hose. A 1.15 m steel probe (inner diameter 4 mm, outer diameter 6 mm) fitted with a sinter metal filter (diameter: 12 mm) at the end, was custom-made at the Technical University of Denmark. The diffusion inlet was covered with 2 layers of air-proof tape to avoid distortion of the measurement results. A special water blocking filter (Gesellschaft Für Gerätebau mbH, Dortmund, Germany) was inserted between probe and detector inlet. To sample silage stacks a hole had to be drilled in the silage. Inserting the probe directly into the stack was generally not possible. The stacks were to compact for the probe to enter more than 20-30 cm. A hand-driven silage drill (ø = 40 mm, length 1.25 m, Frøsalget, Brørup, Denmark) was used to extract silage samples to a depth of 1 m. Afterwards the gas probe was inserted to the bottom of the hole with the probe inlet in the silage. The pump was activated for at least 40 sec until stable readings were obtained from the detector. Between measurements atmospheric air was flushed through the detector for 2-3 minutes, until oxygen concentration was stable at 21%.

Table A-1: Concentrations of O2 and CO2 detected in 20 maize silage stacks sampled in January 2007. The measurements are conducted with a G750 Polytector II, a hand held gas detector.

Farm	O_2	CO_2
1	2.6	14.6
2	0.9	22.4
3	2.8	19.4
4	1.0	24.6
5	1.0	21.6
6	0.6	19.6
7	9.3	10.3
8	1.0	19.6
9	3.3	15.4
10	0.2	28.0*
11	0.3	22.6
12	0.5	26.2*
13	1.5	24.0
14	0.7	28.8*
15	0.2	17.8
16	1.7	13.0
17	0.6	18.6
18	0.6	25.4*
19	1.3	34.5*
20	2.1	17.4
Average	1.6	21.2
Min	0.2	10.3
Max	9.3	34.5
Std.dev.	2.0	5.85

*Above calibration range (>25% CO_2)

Appendix B: Development of an LC-MS/MS method for the detection of 27 mycotoxins in maize silage.

Materials and Methods

Chemicals and reagents

Acetonitrile (Rathburn) was of HPLC grade. Acetic acid (Merck), formic acid (Merck), ammonia water 33% (Merck), ammonium formiat (BDH), natrium acetate and magnesium sulfate, granulate (J.T. Baker) and ammonium acetate (Merck) were all of analytical reagent grade. Water was ultra-purified using a Millipore system (Molsheim France).

Standards were purchased from commercial suppliers; enniatin B from Alexis Biochemicals (Rungsted Kyst Denmark), alternariol, alternariol monomethyl ether, citrinin, deoxynivalenol, fumonisin B1, fumonisin B2, gliotoxin, mycophenolic acid, nivalenol, ochratoxin A, patulin, roquefortine C, T-2 toxin, tenuazonic acid, zearalenone all from Sigma-Aldrich (Broendby, Denmark). Andrastin A (Std vail 512 met 578) and fumitremorgin A were cleaned-up in house from fungal extracts.

Qualitative standards of citreoisocoumarin, fumigaclavine A, fumigaclavine B, fumigaclavine C, fumitremorgin C, marcfortine A, marcfortine B, PR-toxin and roquefortine A from the metabolite collection at Center for Microbial Biotechnology were only in sufficient amount for the LS-MS/MS optimisation. For the spiking experiments a pooled fungal extract was prepared. Agar cultures of *A. tenuissima, P. roqueforti, P. paneum and A. fumigatus* were extracted according to Smedsgaard (1997) with a few modifications. Altersetin was only available in a fungal extract. Its presence in *A. tenuissima* agar extracts was confirmed by LC-MS-(HR) and UV characteristics.

All mycotoxin solutions were prepared in methanol or acetonitrile and kept at –18 °C unless otherwise recommended by the manufacturer. The abbreviations applied to the fungal metabolites are listed in table B-1.

Sample preparation

Silage samples were frozen with liquid nitrogen and homogenised in a blender. Extraction was performed by a modified version of a method for multiple pesticide residues in food known as QuEChERS (Lehotay 2005a, Lehotay 2005b): In a 50 mL plastic tube 10 g sample was extracted with a buffered mixture of 10 ml 1% acetic acid in acetonitrile, 5 mlMilliQ water and 1.67 g sodium acetate trihydrate by shaking for 1-2 minute. Then 4.0 g anhydrous magnesium sulfate was added and the tube was shaken (<10 sec) to obtain phase separation. After 10 min centrifugation (4500 RCF, 25ºC) using a Heraeus sepatech Megafuge 3.0R the upper acetronitrile phase was collected. Before LC-MS/MS analysis the samples were filtered through a 0.45 µm syringe filter in Mini-UniPrep HPLC vials (Whatman).

LC-MS/MS method

HPLC separation of 1 uL injected sample was performed on an Agilent 1100 series from Agilent Technologies (Palo Alto, CA, USA) with a Gemini 3u C6-Phenyl, (3 µm, 2.0 x 100 mm) column equipped with a safety guard cartridge (Gemini C6-Phenyl, 4 x 2.0 mm) both from Phenomenex (Alleroed, Denmark). Samples were analysed in two separate runs, one in positive electrospray

ionization (ESI) mode and one in negative ESI mode. HPLC eluents were prepared daily. The mobile phases were (A) ammonium formiate 0.4 mM, 0.2% formic acid in water (pH = 2.5) and (B) 100% acetonitrile for data recorded in ESI+. In ESI- they were (A) 0.02% formic acid in water and (B) 100% acetonitrile. The gradient conditions were identical. During data collection a flow rate of 0.3 ml/min of was used: From 0 to 4 min 10% B was hold and then going to 100% B from 4 to 22 min. The LC-system was cleaned after each sample. First 20 uL 5% formic acid in acetronitrile was injected and 100%B was kept with 0.5 ml/min for 8 min. Then 20 uL methanol was injected and the gradient and flow rate were changed to 10% B and 0.3 ml/min in 5 min. After injection of 20 uL water equilibrating was allowed for 7 min. This gave a total runtime of 44 min per sample. The auto sampler and column temperature was 25°C.

A Quattro Ultima triple quadrupole MS (Waters, Manchester, UK) with Masslynx v. 4.1 software (Waters) was used for data collection and processing. The MS was tuned so the mass spectra were symmetrical and the width of the top was 1 mass unit. The capillary voltage was 3.0 kV. The source and desolvation temperatures were 120°C and 400°C, respectively. The cone gas flow was 80 l/h and the desolvation gas flow was 530 l/h. Argon was used as collision gas at ~ 2.5 10^{-3} mbar and the electron multiplier voltage applied was 650 V. The transition of one precursor ion into two product ions was recorded in multiple reaction monitoring (MRM) mode. Cone voltage and collision energy for each compound can be seen in table 1. Inter channel delay was 0.02 s and mass range 0.2-0.3 Da. The dwell times was optimised for the individual transitions and in the range 0.1-0.5 s.

The response was calculated as the chromatographic peak area for all compounds, except for PAT where height were used. A linear calibration curve was obtained by plotting the response of the analyte against the concentrations (c), weighted $1/c^2$. The spiking levels were toxin specific and were intended to be near the expected detection limit.

Validation set-up

3 series were performed by two different technicians and on different days. Each series included 3 blind samples, 3 replicates of samples spiked quantitatively at low, medium and high level and 3 replicates of samples spiked with a fixed volume fungal mixture. Maize silages samples from well ensilaged Danish stacks with low toxin content were pooled and used as blank and for spiking. The blank silage had traces of 5 ppb ENN B, which was determined using standard addition. The matrix matched calibration curve of the quantitative standards (n=6) covered 2 decades. One matrix matched fungal standard (n=1) equal to the fungal spike level was included. Standards were analysed twice; in the beginning and at the end of each sequence. From the results obtained repeatability, reproducibility, recovery was calculated for each compound. For compounds quantitatively available the LOD was determined.

Table B-1: Parameters for mass spectrometric detection of 32 fungal secondary metabolites. The quantification ion is listed as the first product ion.

Compound	Abbreviation	RT (min)	Cone (V)	Precursor ion (m/z)	Product ions (m/z)	Collision (eV)
ESI-						
Alternariol	AOH	15.0	35	257.0	214.9, 146.8	25, 30
Alternariol monomethyl ether	AME	16.7	30	271.1	255.8, 227.8	22, 30
Altersetin	ALS	20.0	30	398.2	354, 310.1	22, 23
Andrastin A	AND A	17.8	50	485.3	425.1, 453.1	35, 30
Citreoisocoumarin	CICO	12.3	35	277.1	218.9, 190.9	20, 27
Deoxynivalenol	DON	2.9	15	341.1	265, 295	10, 10
Gliotoxin	GLI	13.6	15	325.0	260.9, 242.9	10, 15
Mycophenolic acid	MPA	15.6	35	319.1	190.8, 178.8	25, 20
Nivalenol	NIV	1.8	18	357.1	281, 310.9	15, 10
Ochratoxin A	OTA	17.8	28	402.1	210.9, 166.7	30, 35
Patulin	PAT	2.5	15	153.0	108.6, 80.6	8, 8
Penitrem A	PEN A	19.5	50	632.3	546, 293.9	30, 50
Roquefortine C	ROQ C	11.9	35	388.2	190, 318	30, 30
Tenuazonic acid	TEA	13.4	30	196.1	111.7, 138.7	25, 18
Zearalenone	ZEA	17.0	30	317.1	130.8, 174.7	30, 25
ESI+						
Citrinin	CIT	16.4	22	251.1	233, 190.9	20, 25
Cyclopiazonic acid	CPA	18.0	40	337.2	195.9, 181.9	30, 25
Enniatin B	ENN B	19.9	30	657.4	314, 527	37, 25
Fumigaclavine A	FUC A	7.1	30	299.2	208, 239	28, 18
Fumigaclavine B	FUC B	2.1	30	257.2	192, 167	30, 27
Fumigaclavine C	FUC C	12.2	40	367.2	238, 307	30, 20
Fumitremorgin A	FUT A	19.8	15	602.3	460, 498	15, 15
Fumitremorgin C	FUT C	15.5	30	380.2	324, 211.7	20, 35
Fumonisin B1	FB1	12.6	40	722.5	334, 528	38, 30
Fumonisin B2	FB2	13.4	40	706.6	336, 318	35, 35
Marcfortine A	MAC A	12.0	40	478.3	419, 450	35, 25
Marcfortine B	MAC B	11.7	20	464.0	436, 419	22, 30
Mevinolin	MEV	18.5	40	405.3	225, 172.6	20, 23
Mycophenolic acid	MPA	15.5	20	321.1	207, 159	20, 40
Ochratoxin A	OTA	17.2	20	404.1	357.7, 341	15, 20
PR-toxin	PR	15.8	15	321.1	260.9, 278.9	10, 15
Roquefortine A	ROQ A	10.0	25	299.18	239, 196.9	18, 25
Roquefortine C	ROQ C	12.6	25	390.2	322, 334	22, 30
Sterigmatocystin	STE	17.3	40	325.0	280.9, 309.9	35, 28
T-2 toxin	T-2	15.9	30	484.3	214.9, 305.0	20, 20

Appendix C: Pilot experiment on silage monitoring

One silage stack located at Freerslev Kotel I/S, Gørløse, Denmark was visited 5 times from December 2005 until July 2006. At each visit one silage sample was extracted with a 1.25 m long silage drill and the cutting face was examined for fungal hot-spots. Filamentous fungi were isolated after cultivation on V8 and DG18 and identified.

The detected species at each sampling time are presented in Table C-1.

Table C-1: Species of filamentous fungi isolated from the same silage stack at different times of the year.

20/12	**26/1**	**6/3**	**16/5**	**13/7**
-	-	*P. roqueforti* *P. paneum*	*P. roqueforti* *P. paneum* *Penicillium* sp. *Mucor* sp.	*P. paneum* *B. nivea* *Mucor* sp. Coelomycet

Appendix D: Popular scientific papers in Danish

D-I Ulf Thrane, Ida ML Drejer Storm and Jens L Sørensen (2006) Svampe og deres toksiner i majsensilage. KvægInfo, no. 1633. Available on the internet: http://www.lr.dk/kvaeg/system/visforskningsresultater.asp?category=kvforsk

Ulf Thrane, Ida M.L. Drejer Storm & Jens Laurids Sørensen
Center for Mikrobiel Bioteknologi (CMB)
BioCentrum-DTU
Danmarks Tekniske Universitet
2800 Kgs. Lyngby

Svampe og deres toksiner i majsensilage

Svampesammensætningen i dansk majs og majsensilage vil blive undersøgt med henblik på at vurdere potentielle mykotoksiner i kvægfoder. Det sker som en del af samarbejdsprojektet "Mycotoxin carry-over from maize silage via cattle into dairy products" (2005-2009) finansieret af Direktoratet for FødevareErhverv. De indledende svampeundersøgelser af majs fra høsten 2005 viser at der ud-over de forventede Fusarium graminearum og F. culmorum, også var en høj frekvens af F. avena-ceum og F. equiseti. Fusarium avenaceum var faktisk den hyppigst isolerede art og interessant nok danner den ingen af de mykotoksiner man normalt screener for (trichothecener, fumonisin og zeara-lenon), men derimod moniliformin og enniatiner. Deres forekomst i dansk majs er ukendt, men vil blive undersøgt. Endvidere er der fundet en høj frekevns af Alternaria arter i majsprøverne og deres betydning for majsens kvalitet vil også blive undersøgt, da dette er ukendt. I selve majsensilagen er det helt andre svampe der forekommer. Her er der fundet Penicillium roqueforti, P. paneum, P. commune, Monascus ruber, Aspergillus flavus, Byssochlamys nivea og Mucor arter. Toksiner fra disse svampe er ikke omfattet af de etablerede overvågnings- og screeningsprogrammer, men fra enkelte analyser vides det at deres toksiner kan forekomme i majsensilage til kvægfoder. De toksikologiske konsekvenser er dog ukendte. I vores del af projektet vil der blive udviklet hurtige analysemetoder til at detektere de relevante svampearter og svampenes fysiologiske og kemiske egenskaber under mark og ensilageforhold vil blive undersøgt. Endvidere vil vi i samarbejde med dyrefysiologer, toksikologer og analysekemikere vurdere svampetoksinernes effekt på malkekvæg og mulige overførsel til levnedsmidler.

Majsensilage mistænkes for at kunne indeholde en række mykotoksiner som er blevet produceret i majsplanten under svampeangreb, enten mens den groede på marken eller under ensileringen. Det har længe været kendt, at nogle mykotoksiner er kræftfremkaldende, mens andre er cytotoksiske eller giver hormonale og neurologiske påvirkninger. Netop derfor er mykotoksinerne under mistanke for at være årsagen til tilfælde af dårlig trivsel, nedsat mælkeproduktion og dødsfald, der har været observeret blandt danske besætninger af malkekvæg. Der har også været diskussioner og spekulationer om hvorvidt toksinerne kan overføres til kvægets blod og mælk, og derigennem havne på vores spiseborde.

For at belyse disse problemer har Direktoratet for FødevareErhverv under forskningsprogrammet Fremtidens Fødevaresektor finansieret et samarbejdsprojekt *"Mycotoxin carry-over from maize silage via cattle into dairy products"*, der startede sommeren 2005 og skal løbe indtil 2009. Partnerne er Danmarks Fødevareforskning, Danmarks JordbrugsForskning, Dansk Landbrugsrådgivning, Plantedirektoratet samt CMB/BioCentrum-DTU. Hertil kommer et PhD projekt ved CMB finansieret af Dansk Kvæg, Forskerskolen FOOD (Levnedsmiddelcenteret) og DTU.

På CMB vil vi fastslå hvilke svampearter der hyppigst optræder i majs før og under ensilering ved at undersøge majs- og ensilageprøver indsamlet af samarbejdspartnerne. Denne artssammensætning kaldes den majsassocierede funga. Prøverne vil sideløbende blive undersøgt for indholdet af kendte mykotoksiner for at identificere mulige årsager til forekomster af sygdomstilfælde i danske kvægbesætninger. På CMB vil vi yderligere undersøge hvordan fungaen ændrer sig over tid i en ensilagestak, hvilket er et vigtigt aspekt da dele af en ensilagestak kan ligge i op til omkring et år, før den anvendes.

På marken
På grund af den lange vækstsæson i Danmark er risikoen for svampeinfektioner, mens majsen gror på marken, meget høj. De to vigtigste mykotoksinproducerende svampeslægter, som kan inficere majs inden ensilering, er *Alternaria* og *Fusarium*. Af disse to svampeslægter har fokus primært været på *Fusarium* og specielt *Fusarium* mykotoksinerne deoxynivalenol (DON), nivalenol (NIV) og zearalenon har været under

mistanke for at være skyld i nogle af de problemer, som landmænd har oplevet i forbindelse med fodring af majsensilage.

Klimaet bestemmer hvilke svampe der trives

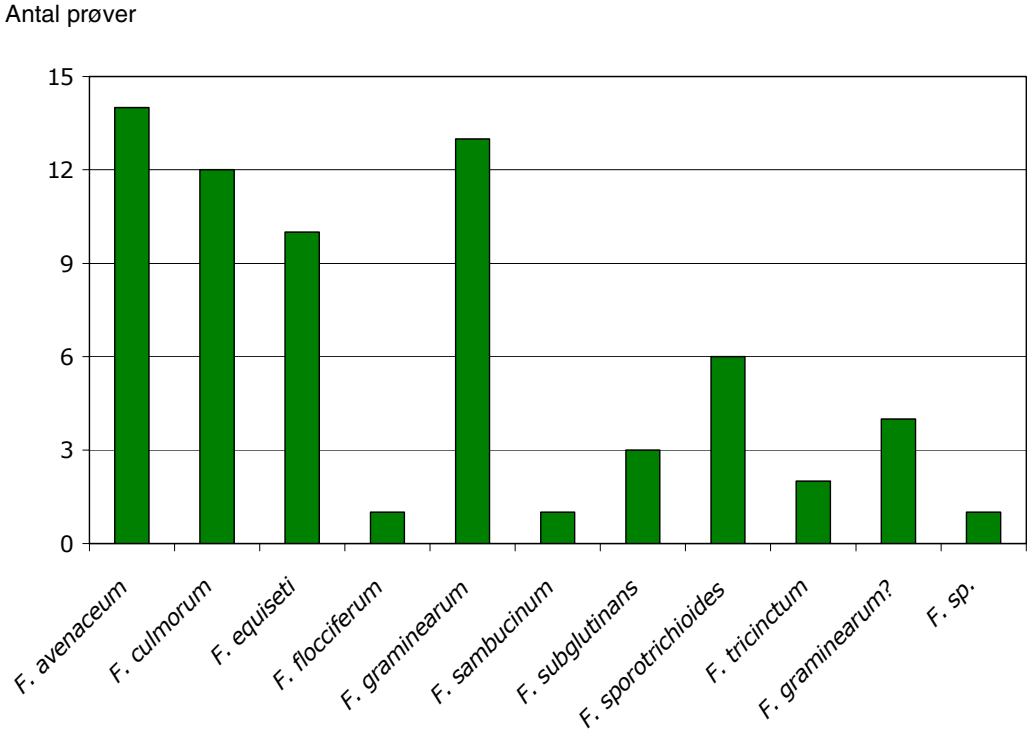
Fra udenlandske studier ved vi, at en række svampe kan angribe majsen, mens den gror på marken. Disse studier er dog hovedsagelig foretaget i lande med varmere klima, hvilket bevirker at resultaterne ikke kan overføres direkte til danske forhold. I disse lande er hovedproblemet aflatoksin og fumonisin, som bliver produceret af henholdsvis *Aspergillus flavus* og *Fusarium* arterne *F. verticillioides* og *F. proliferatum*. Disse mykotoksiner er blandt andet kræftfremkaldende. De ansvarlige svampe kan dog ikke klare sig særlig godt i det kølige danske klima og er derfor ikke et problem i dansk produceret majs.

Flere *Fusarium* arter forekommer

Studier i Tyskland, der har et bedre sammenligneligt klima med Danmark, viser at de hyppigst forekommende *Fusarium* arter er *F. graminearum*, *F. culmorum* og *F. equiseti* som bl.a. kan lave trichothecener (inkl. DON og NIV) og zearalenon (Baath *et al*. 1990). DON, også kendt som vomitoksin, kan have antibiotiske effekter mod mikroorganismer i vommen og derved hæmme foderoptagelsen hos kvæg. Zearalenon passerer derimod gennem vommen til tyndtarmen og optages i blodet, hvor det virker østrogenforstyrrende, hvilket blandt andet kan resultere i fertilitetsproblemer (Dänicke *et al.* 2005).

Mykotoksiner fra de undersøgte *Fusarium* arter overstiger ikke grænseværdierne

Forekomsten af DON, NIV, zearalenon og fumonisin samt de mindre udbredte T-2 toxin og HT-2 toxin er blevet undersøgt i majsprøver indsamlet fra hele Danmark, for at finde ud af hvorvidt disse mykotoksiner er et reelt problem (Cordsen *et al.* 2006). I undersøgelsen var DON det hyppigst forekommende mykotoksin og kunne detekteres i 97 % af alle prøver, dog uden at overstige den vejledende grænseværdi på 5 mg DON/kg. Indholdet af de andre mykotoksiner oversteg heller ikke de respektive vejledende grænseværdier, dog fandtes der i 3 ud af 66 prøver et T-2 toxin eller HT-2 toxin indhold der overskred 0,1 mg/kg der har været foreslået som en kritisk værdi. Konklusionen af undersøgelsen er derfor at disse mykotoksiner ikke udgør et akut problem i majs dyrket i Danmark.



Figur 1. Fusarium fund i 30 danske majsprøver

Andre mykotoksiner fra *Fusarium* arter vil blive undersøgt

For at undersøge om andre mykotoksiner produceret af *Fusarium* kan udgøre en risiko, har vi ved CMB lavet en foreløbig kortlægning af *Fusarium* arter i majs i Danmark. Resultaterne viser at den hyppigst

forekommende art er *F. avenaceum* (se figur 1), der ikke kan danne nogle af de ovennævnte mykotoksiner, men som derimod producerer moniliformin og enniatiner. Vi vil screene de indsamlede majsprøver for moniliformin og enniatiner og derved fastslå om disse mykotoksiner udgør en risiko for kvæg i Danmark.

Også *Alternaria* undersøges
Derudover ønsker vi at undersøge om den anden hyppigt forekommende svampeslægt, *Alternaria,* danner mykotoksiner i majs i Danmark som kan udgøre et problem. *Alternaria* en et af de hyppigst optrædende patogener i korn og majs i tempererede egne inkl. Danmark (Andersen *et al.* 1996; Müller 1991). Der findes dog ikke nogle undersøgelser af *Alternaria* mykotoksiner i majs, selv om vi ved at *Alternaria* arter kan producere en række stoffer, der kan være skadelige for kvæg.

I ensilagen
Når majsen ensileres ændres vækstbetingelserne for svampe dramatisk. Den smule ilt der er tilbage i den velpakkede ensilage forbruges hurtigt, der dannes CO_2 i stedet og pH falder, når mælkesyrebakterier danner blandt andet mælkesyre og eddikesyre. De svampearter, der er groet frem i marken, kan derfor ikke klare sig og vil ikke kunne vokse videre, men deres eventuelle mykotoksiner vil kun i meget ringe grad påvirkes af de ændrede forhold.

Svampevækst ses hyppigst på overfladen
Andre svampearter er mere resistente mod lave ilt-koncentrationer, høje CO_2-koncentrationer og lav pH. Ind i mellem får de fodfæste i ensilagen og kan danne svampekolonier. I en ideel ensilage udgør kombinationen af lav ilt-koncentration, høj CO_2 og lav pH en effektiv hindring for væksten af svampe. Svampevækst ses derfor typisk på overfladen af stakken, hvor ilt-koncentrationen er højere, CO_2-koncentrationen lavere og mikrobiel omsætning af mælkesyre har hævet pH.

Svampearter der i større eller mindre grad kan tolerere ensilageforhold er *Penicillium roqueforti*, *Monascus ruber*, *Aspergillus fumigatus*, *Byssochlamys nivea*, *Geotrichum candidum*, *Mucor* og *Trichoderma* arter. Disse svampearter er isoleret fra tysk, fransk og hollandsk majsensilage (f.eks. Garon *et al.* 2006).

Tabel 1. Svampearter fundet i 38 danske majsensilageprøver indsamlet 20/4-05 – 21/9-05

Art	Forekomst (antal prøver)
Penicillium roqueforti	14
Byssochlamys nivea	7
Monascus ruber	7
Zygomyceter	11
Aspergillus flavus	3
Penicillium paneum	5
Øvrige *Penicillium*	5
Eurotium spp.	5

Flere problematiske svampe er fundet i majsensilage
Penicillium roqueforti er hyppigt forekommende i majsensilage (Tabel 1) og observeres både på overfladen af stakke og i klumper eller lag inde i dem. Denne svamp er blandt andet i stand til at producere PR-toxin, mycophenol syre, marcfortin og roquefortin C. PR-toxin er akut toksisk, mens giftigheden af de øvrige ikke er vel bestemt. *Monascus ruber* er kendt for at producere citrinin og monakoliner. Citrinin kan give nyre- og leverskader og således være direkte skadeligt. Desuden har begge stoffer antimikrobiel aktivitet og kan derved påvirke vomfunktionen hos drøvtyggere. En tysk undersøgelse af 135 majsensilageprøver fandt begge stoffer i lave koncentrationer (Schneweis *et al*. 2001). *Aspergillus fumigatus* er kendt for produktion af bl.a. gliotoxin, verruculogen, fumagillin og helvolin syre. Desuden kan indtagelse af spore fra svampen

forårsage aspergillose i dyr og mennesker. De toksikologiske effekter af *A. fumigatus* er således talrige. Ekstrakter af *A. fumigatus* er også vist at have en negativ effekt på forgæringen i vommen (Morgavi *et al*. 2004). *Byssochlamys nivea* producerer patulin og byssochlamin syre. Patulin er akut toksisk og kræftfremkaldende (Scudamore & Livesey 1998) og er fundet i ensilage i koncentrationer op til 40 ppm (Escoula 1974). *Geotrichum candidum* og *Mucor* arter er ikke associeret med nogle toksiske metabolitter. Førstnævnte kan dog udskille stoffer, der gør ensilage ildelugtende. *Trichoderma* arter kan danne mange toksiske og biologisk aktive stoffer men direkte toksikoser er sjældent observeret.

Inficerede ensilageprøver vil blive undersøgt

Vi har indledningsvis undersøgt et mindre antal danske majsensilageprøver med svampevækst. Fra disse prøver er isoleret *Penicillium roqueforti, P. paneum, P. commune, Monascus ruber, Aspergillus flavus, Byssochlamys nivea* og *Mucor* arter. Dette billede stemmer således pænt overens med de tidligere undersøgelser. Der skal dog skelnes mellem detektion af svampe, der aktivt vokser i ensilage og svampe, der er i stand til at overleve for eksempel i form af sporer og derfor ville kunne detekteres ved en mykologisk analyse. Det er dog ikke altid muligt at vurdere, om der i litteraturdata er skelnet mellem disse forhold. Vi planlægger derfor at isolere og identificere de aktivt voksende svampe i dansk majsensilage ud fra inficerede ensilageprøver, såkaldte "hot spots".

Udvikling af analyse rettet mod praksis

Med de mange toksiner, der kan produceres af ensilagesvampe, kombineret med de toksiner der stammer fra majsen før ensilering, er det klart at analyse for mykotoksiner ikke er en simpel opgave. Toksinerne har forskellige kemiske og fysiske egenskaber og kræver forskellige oprensnings- og analysemetoder. Endnu et mål er derfor at inddele toksinerne i nogle få grupper og udvikle analysemetoder til detektion af disse overordnede grupper.

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Links:

http://www.biocentrum.dtu.dk/studievalg_forside/historier/25_ensilage.aspx

http://www.dffe.dk/Default.asp?ID=19910

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Forekomst af skimmelsvamp i majsensilage

Undersøgelser af majsensilage viser, at den højeste koncentration af levedygtig skimmelsvamp i ensilage findes i det tidlige forår. Det tyder også på, at mug i ensilage generelt opstår i forbindelse med åbning af stakkene eller beskadigelse af forseglingen.

Vækst af skimmelsvampe i ensilage er et hyppigt problem. Det giver forringelse af foderværdien, tab af biomasse ved mikrobiel nedbrydning, og medfører risiko for kontaminering af foderet med mykotoksiner. Som led i et større projekt har danske forskere fra Det Jordbrugsvidenskabelige Fakultet, Danmarks Tekniske Universitet og Dansk Kvæg undersøgt majsensilage for forekomsten af skimmelsvampe gennem fodringssæsonen 2007.

Sæson for muggen ensilage?

I alt 20 tilfældigt udvalgte malkekvægsbesætninger blev besøgt i januar, marts, maj, juli og september 2007. Ved hvert besøg blev der udtaget en boreprøve af den stak majsensilage, der var i brug. Boreprøven blev taget i fuld dybde 1 meter bag snitfladen. Efter neddeling blev en del af denne boreprøve anvendt til bestemmelse af antallet af kolonidannede skimmelsvampe, mælkesyrebakterier og gær. Al vækst af skimmelsvamp på snitfladen blev registreret og prøver af disse "hot-spots" blev indsamlet.

Mest skimmelsvamp i foråret

Resultaterne fra de mikrobielle undersøgelser af ensilageprøverne viser, at der er store variationer mellem besætninger og fra gang til gang i de enkelte besætninger. Det gennemsnitlige antal af mælkesyrebakterier og gær er stabilt over tid, men der er signifikant flere kolonidannende skimmelsvampe i marts måned. Antallet af indsamlede hotspots er også højest i maj og markant lavere i september end ved de fire første indsamlinger.

Stort set alle ensilager indeholder skimmelsvamp - selv fra en sund og velpasset ensilage kan der dyrkes skimmelsvamp. Men svampenes levedygtighed falder tilsyneladende efter længere tids ophold i ensilagens sure og ilt-fattige miljø. Disse resultater tyder på, at hvis ensilage, der først skal bruges sent på opfodringssæsonen, holdes tæt forseglet så længe som muligt, mindskes risikoen for vækst af skimmelsvamp efter åbning af stakken.

Yderligere analyser af de indsamlede prøver og data vil afklare hvilke svampe og eventuelle toksiner, der findes i ensilagen, og klarlægge, om der er en sammenhæng mellem forekomsten af skimmelsvamp og fysiske og kemiske egenskaber ved ensilage og stakke. Denne del af projektet afsluttes juni 2009.

Flere oplysninger:

Ida ML Drejer Storm, CMB,
Danmarks Tekniske Universitet
Tlf.: 4525 2608
E-mail: id@bio.dtu.dk

Rudolf Thøgersen, Dansk Kvæg
Tlf.: 8740 5317
E-mail: rut@landscentret.dk

Ny KvægForskning udgives af Kvægbrugets Forsøgscenter og Aarhus Universitet, Det Jorbrugsvidenskabelige Fakultet i samarbejde med Dansk Kvæg

Redaktion
Christian Børsting, ansv.,
Kirsten Foss Marstal,
Linda S. Sørensen,
Karin Smedegaard, sek.
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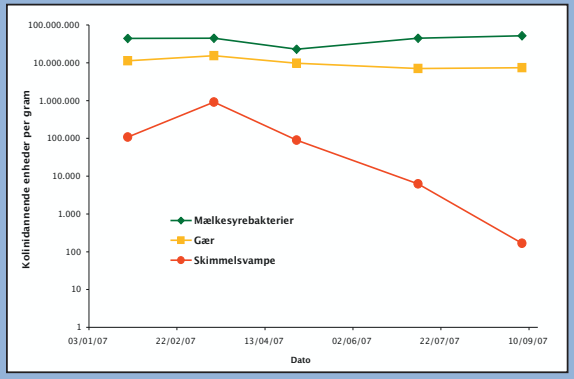
Layout og tryk:
Rounborgs Grafiske Hus,
Holstebro (23498)

ISSN 1603-600X
ISSN 1903-2390 (elektronisk)



Gennemsnitligt antal levedygtige mælkesyrebakterier, gær og skimmelsvampe per gram ensilage ved de 5 indsamlinger i januar-september 2007.

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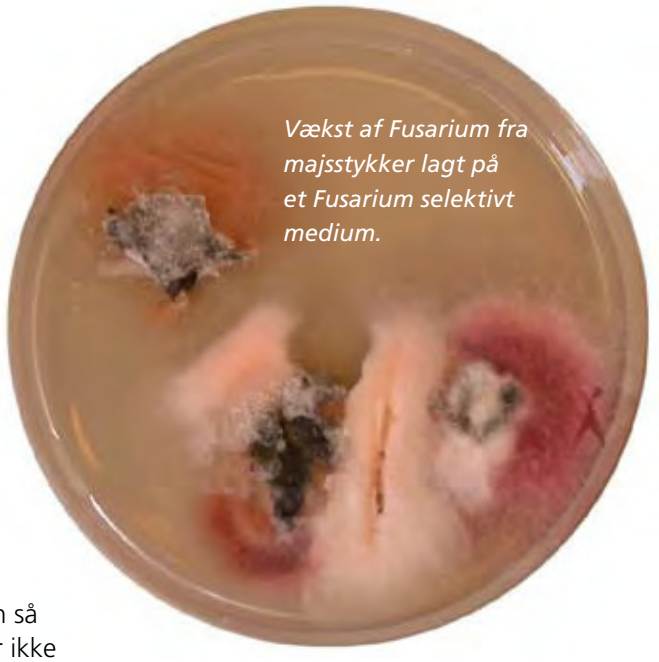
Marksvampes mykotoksiner i majs

- nok ikke skyld i sundheds-problemer hos kvæg



Vækst af Fusarium fra majsstykker lagt på et Fusarium selektivt medium.

Der har været en del spekulationer om, hvorvidt mykotoksiner i majsensilage er skyld i de sundhedsmæssige problemer, der er set i nogle danske kvægbesætninger. Ud fra undersøgelser af tre svampegrupper, som ender op i ensilagestakkene, er der indtil videre ikke noget der tyder på, at disse enkeltvis er årsagen.

Mange forskellige skimmelsvampe er i stand til at inficere majsplanter, mens de gror på marken. Udover at disse svampe kan forårsage et betydeligt udbyttetab, er der også en risiko for, at de kan danne forskellige skadelige mykotoksiner.

Uskadelige niveauer af mykotoksiner

Fusarium er den vigtigste mykotoksindannende svampeslægt, som er i stand til at inficere majsplanter, mens de gror på marken. Forskellige *Fusarium* arter danner mange og meget forskellige mykotoksiner. Forekomsten af udvalgte mykotoksiner er tidligere blevet undersøgt i et samarbejde mellem Dansk Landbrugsrådgivning og Det Jordbrugsvidenskabelige Fakultet (Flakkebjerg). Resultaterne af disse undersøgelser viser, at selvom mykotoksinerne forekommer hyppigt, så er niveauerne så lave, at de højst sandsynligt ikke udgør en risiko. På Danmarks Tekniske Universitet har vi udviklet metoder til at undersøge nogle flere mykotoksiner, moniliformin og enniatiner, som dannes af andre *Fusarium* arter. Vores resultater viser, at selvom også disse mykotoksiner forekommer hyppigt, så er niveauerne igen så lave, at de sandsynligvis heller ikke er skadelige.

Ny gruppe svampe i majsmarken undersøgt

Der findes endvidere en anden gruppe svampe, udover *Fusarium*, som kan inficere majs på marken. Slægterne *Alternaria* og *Phoma,* som vi hyppigt har isoleret fra frisk majs, danner også mykotoksiner. Disse mykotoksiner er kemisk forskellige fra dem, som *Fusarium* danner, og har ikke været omfattet af tidligere undersøgelser. Vore foreløbige resultater har dog vist, at mykotoksiner fra disse to slægter heller ikke udgør en risiko.

Svampeart inficerer ensilage ved pakning

Problemer med skimmelsvampe er dog ikke overstået, når majsen er høstet og blevet ensileret, idet en tredje gruppe af mykotoksindannende svampearter inficerer ensilagestakkene, når de pakkes. Arter af *Penicillium* vokser som blågrønne klumper i nogle ensilagestakke, mens vækst af *Monascus* ses som røde bræmmer eller klumper i andre stakke. På Danmarks Tekniske Universitet er vi i øjeblikket i gang med at undersøge konsekvenserne af svampenes vækst og deres dannelse af mykotoksiner i ensilagestakkene.

Meget tyder på, at mykotoksinerne fra de tre svampegrupper, som ender op i ensilagestakkene, enkeltvis *ikke* er årsagen til problemer i kvægbesætningerne. Dog findes der ingen undersøgelser, som ser på den samlede effekt af den cocktail af mykotoksiner, der kan være til stede i majsensilagen.

Flere oplysninger

Jens Laurids Sørensen, DJF,
Aarhus Universitet, tidligere DTU
JensL.Sorensen@agrsci.dk
tlf.: 8999 1133

Ida ML Drejer Storm,
Birgitte Andersen og
Ulf Thrane,
Danmarks Tekniske Universitet (DTU), Institut for Systembiologi, 2800 Kgs. Lyngby

Original paper I

Storm, I.M.L.D., Sørensen, J.L., Rasmussen, R.R., Nielsen, K.F., and Thrane, U. (2008) Mycotoxins in silage. *Stewart Postharvest Review* **6**(4).

Stewart Postharvest Review
An international journal for reviews in postharvest biology and technology

Mycotoxins in silage

Ida ML Drejer Storm,[1*] Jens Laurids Sørensen,[1] Rie Romme Rasmussen,[2] Kristian Fog Nielsen[1] and Ulf Thrane[1]
[1]Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, Kgs. Lyngby, Denmark
[2]The National Food Institute, Technical University of Denmark, Søborg, Denmark

Abstract
Purpose of review: This paper reviews the present knowledge on mycotoxins in silage, focusing on grass and maize silage. This includes the occurrence of filamentous fungi pre- and postharvest, possible and confirmed mycotoxins in silage, toxicological concerns and means to prevent the problem.
Findings: Preharvest contamination of grass and maize by *Fusarium*, *Aspergillus* and *Alternaria* can lead to contamination of silage. Well known mycotoxins deoxynivalenol (DON), zearalenone (ZEA), fumonisins and aflatoxins have been detected in silages but concentrations seldom exceed regulatory limits. It also appears that DON, ZEA and fumonisins are degraded in silage, but exact mechanisms are unknown. Postharvest spoilage is dominated by *Penicillium roqueforti*, *Aspergillus fumigatus* and Zygomycetes. Both *P. roqueforti* and *Asp. fumigatus* produce a wide range of secondary metabolites, some of them confirmed mycotoxins, others with antimicrobial or immunosuppressive effects. Some fungal metabolites have been detected in silage but many have not been looked for. Evidence for acute toxicosis caused by contaminated silage is rare. Mycotoxins in silage are more often associated with less specific symptoms like ill-thrift or decreasing yield. This may be caused by long-term exposure to the complex mixture of secondary metabolites that silage can contain. Mycotoxins with antimicrobial effects may also affect ruminant digestion. To prevent postharvest spoilage of silage the most important factor is omission of oxygen. Additives can improve certain silage properties but they are not conclusively an advantage and cannot replace good silage management.
Directions for future research: The effects of long-term exposure and of complex mixtures of bioactive fungal compounds are subjects of interest. Especially high-yielding livestock may be subject to sub-acute symptoms under these conditions. There is also a need for analytical methods with specificity and accuracy to determine many of the less known mycotoxins and secondary metabolites in silage as well as possible unknown compounds.

Keywords: silage; grass; maize; mycotoxins; preharvest; postharvest

Abbreviations

DAS	Diacetoxyscirpenol
DON	Deoxynivalenol
ELEM	Equine Leukoencephalomalacia
FB1	Fumonisin B_1
LC-MS	Liquid Chromatography–Mass Spectrometry
NIV	Nivalenol
PPE	Porcine Pulmonary Oedema Syndrome
ZEA	Zearalenone
ZOL	Zearalenol

***Correspondence to:** Ida ML Drejer Storm, Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, Søltofts Plads 221, DK-2800 Kgs. Lyngby, Denmark. Tel: +45 45 25 26 08; Fax: +45 45 88 41 48; email: id@bio.dtu.dk

Stewart Postharvest Review 2008, 6:4
Published online 01 December 2008
doi: 10.2212/spr.2008.6.4

Introduction

Ensiling is used worldwide as a simple and effective way to preserve forage for livestock, using a natural lactic acid fermentation of the feedstuffs which is acidified and can subsequently be stored for long periods without degrading. In modern agriculture, large amounts of silage are consumed each day all year round with dairy cows eating up to 40 kg/day. In Western Europe the total area of land harvested for silage has remained stable at around 15 million hectares since 1990 and worldwide the silage production has tended to increase from 1989–2000 [1]. The use of especially maize silage has increased over the last few decades with the availability of short season maize varieties suitable for temperate climates [1, 2]. In Denmark the production has increased by 500% from 1990 to 2007 [3] and maize silage is very widely used for cattle in both dairy and meat production.

The most common silage crops are grass and maize, but many other products like whole-crop barley, alfalfa, clover, sugar-beet tops, and residues from sugar production can be preserved as silage [4**]. A thorough review of silage making from crops to nutritive value is covered by McDonald *et al.* [4**]. In all cases the product is harvested, cut in suitable sizes and packed tightly in either silos, stacks or bales, and sealed to avoid oxygen infiltration. Residual enzymatic activity of the plant and microbial respiration of the carbohydrates released by chopping quickly depletes the small amount of O_2 in the stack and raises the concentration of CO_2. One hour after ensilage, O_2 levels in the range from 1 to 2% and CO_2 from 20 to 90% were recorded in baled silage [5]. This selects for the proliferation of natural lactic acid bacteria, whose numbers increase from below 10^2–10^5 CFU/g on plants in the field to 10^9–10^{10} CFU/g in silage that is only a few days old [4**, 6]. They ferment sugars to primarily lactic acid and acetic acid, lowering pH to ~4 or less. Clamp and baled grass silages have been reported to have an average pH of 4.0 and 4.8, respectively [5]. The combination of low O_2 concentration, high CO_2 concentration and a low pH makes silage a very hostile environment for spoilage organisms including bacteria, yeasts and filamentous fungi.

Nevertheless, growth of filamentous fungi is frequently observed in silage. This constitutes a loss of nutritive value for the farmer and, much worse, a risk for contamination with mycotoxins. Toxins in the feed may constitute a health risk for animals and there is also the risk for carry-over to humans via milk and meat [7*]. In the last 30 years, cases of ill-thrift, disease and death in livestock have been related to the presence of mycotoxins in silage [8*–12] and the issue is much debated [2, 7*, 13–18**]. Infestation and subsequent mycotoxin production may take place both pre- and postharvest and silage can thus be contaminated with both well known *Fusarium* toxins like deoxynivalenol (DON) and zearalenone (ZEA), as well as less known secondary metabolites from species of *Penicillium* and other fungi.

This article reviews the present knowledge on mycotoxins in silage including pre- and postharvest contaminants, toxicological issues and means for preventing the problem. The focus is on grass and maize silage for cattle as these are considered the economically most important use of silage crops.

Toxigenic field fungi

The three most important toxigenic genera occurring preharvest in cereals and maize are *Aspergillus*, *Fusarium* and *Alternaria* (Table 1). *Alternaria* and *Fusarium* are often categorised as field fungi whereas some species of *Aspergillus* can occur both pre- and postharvest. The occurrence of these fungi is influenced by several factors, including agricultural practices (crop rotation, crop variety, fertilisation and cultivation methods) and climatic conditions (temperature and moisture).

Small-spored *Alternaria* are common pathogens of small grains and maize with *Alt. alternata, Alt. arborescens, Alt. infectoria* and *Alt. tenuissima* as the predominant species [19]. *Alt. alternata* may not be as common as the literature indicates, as it is often mis-identified. Of these species *Alt. infectoria* is the only one with a known sexual stage (*Lewia*). The infections often occur in the late growth season as black spots on the host plants.

The two predominant toxigenic field *Aspergillus* species are *Asp. flavus* and *Asp. parasiticus*. These two species are mainly found in warm arid, semi-arid and tropical regions and cause huge problems in the Midwestern corn belt in the USA [20]. They can infect growing maize and produce mycotoxins preharvest but may apparently also survive the ensiling process, as findings of *Asp. flavus* in silages have been reported [21–23*].

Species of the anamorphic genus *Fusarium* are destructive pathogens responsible for several diseases including red/pink ear rot of maize and head blights of wheat. In areas with temperate climate, *F. avenaceum, F. culmorum* and *F. graminearum* (teleomorph: *Gibberella zeae*) are the predominant species, whereas the members of the *Liseola* section *F. proliferatum*, *F. subglutinans* and *F. verticillioides* (teleomorph: *Gib. moniliformis*) dominate in warmer parts of the world [24].

Several additional producers of bioactive secondary metabolites are often associated with cereal and maize including species of *Epicoccum, Cladosporium, Diplodia* and *Phoma*. The natural occurrence of mycotoxins produced by these genera in food and feeds has not been studied yet and an estimation of their importance is therefore not possible. *Diplodia* toxins have however been suggested as the primary cause in an Argentinean case where 10 heifers died from eating mouldy maize infected with *Diplodia maydis* [25]. Attention should therefore be given in the future to mycotoxins produced by other genera than *Aspergillus* and *Fusarium*.

Several species of the sexual genus *Epichloë* (anamorph: *Neotyphodium*) are endophytes of some varieties of pooid

Table 1. The most common species of *Fusarium, Alternaria* and *Aspergillus* in preharvest silage crops, some known secondary metabolites and secondary metabolites confirmed in silage.

Species	Secondary metabolites	Detected in silage
Fusarium culmorum, *F. cerealis* and	Culmorin	
F. graminearum	Deoxynivalenol	+
	3- or 15-Acetyl deoxynivalenol	+
	Nivalenol	+
	Fusarenone-X	
	Fusarins	
	Zearalenones	+
	2-Acetylquinazolinone	
	Aurofusarin, Rubrofusarin, Butenolide, Chrysogine	
F. proliferatum, F. subglutinans and	Beauvericin	+
F. verticillioides	Fumonisins	+
	Fusaproliferin (*F. pro*. and *F. sub*.)	
	Fusapyrone (*F. pro*), Fusaric acid	
	Moniliformin (*F. pro.* and *F. sub.*)	
	Naphthoquinone pigments	
F. poae and *F. sporotrichioides*	Aurofusarin	
	Beauvericin	+
	Chrysogine (*F. sporotrichioides*)	
	Culmorin	
	Scirpentriol	+
	Monoacetoxyscirpentriol	+
	Diacetoxyscirpentriol	
	Enniatins	+
	Fusarenone-X (*F. poae*)	
	T-2 toxin	
	HT-2 toxin	+
	Neosolaniol	
	Nivalenol (*F. poae*)	+
F. avenaceum and *F. tricinctum*	2-Amino-14,16-dimethyloctadecan-3-ol (*F. ave*.)	
	Acuminatopyrone (*F. ave*.), Antibiotic Y, Aurofusarin	
	Beauvericin	+
	Butenolide	
	Chlamydosporols	
	Chrysogine	
	Enniatins	+
	Fusarins, Gibepyrone A, Moniliformin, Visoltricin (*F. tric*)	
F. equiseti	Nivalenol	+
	Scirpentriol, monoacetoxyscirpentriol	+
	Diacetoxyscirpentriol, Equisetin, Fusarenone-X	
	Fusarochromanone, Chrysogine	
Alternaria alternata, Alt. arborescens	AAL-toxins (*Alt. arborescens*)	+[a]
and *Alt. tenuissima*	Alternariols, Altertoxins, Tentoxin, Tenuazonic acid	
Alternaria infectoria	Infectopyrones, Novae-zelandins	
Aspergillus flavus and Asp. parasiticus	Aflatoxin B_1 and B_2	+
	Aflatoxin G_1 and G_2 (*A. parasiticus*)	
	Aspergillic acid	
	Cyclopiazonic Acid	+
	Kojic acid	
	Sterigmatocystins	+
	Versicolorin and precursors, 3-Nitropropionic acid	
	Aflavinine, Aflatrem	

[a]Needs reconfirmation

Figure 1. Ball of maize silage infected with *Penicillium roqueforti*, which was observed in the middle of a well managed silage stack.

grasses [26]. *Epichloë* species can infect plants through wounds or stigmata and by seed-transmission whereas the asexual *Neotyphodium* species are only seed-transmitted. The endophytes colonise host plants systematically without causing disease symptoms. Several bioactive alkaloids can be produced by the endophytes during the infections, which are beneficial to the host plant as they can be active against feeding insects or herbivores. Other fitness improvements that may be attributed to the symbiosis between host plants and endophytes include growth stimulation and enhanced drought tolerance [27]. Some neurotoxic alkaloids have been implicated in livestock toxicosis, including ergot alkaloids [28] and lolitrems [29*]. For reviews on this subject see [16, 18**] The recognised toxicosis events occurred when livestock had been feeding on *Epichloë* or *Neotyphodium* infected grass, but so far nothing is known about their occurrence and stability in grass silage.

Postharvest contamination

The ensiling process eliminates most fungi from the field [30*, 31]. There are however other species of filamentous fungi that are able to tolerate both organic acids, carbon dioxide and the low availability of oxygen (Table 2).

The most commonly found filamentous fungi in silage are *Penicillium roqueforti* and the closely related *P. paneum* [22, 32*, 33–35]. *P. roqueforti* has its pH optimum between pH 4 and 5 [36], tolerates high levels of CO_2 [37] as well as the different organic acids commonly found in silage [22, 38]. The optimum temperature is 25°C but *P. roqueforti* may grow at 5°C [38]. Thus it is able to grow in silage all year round, even in temperate climates. *P. roqueforti* also sporulates heavily and spores are almost always present even in healthy looking maize silage [Storm IMLD, unpublished]. Growth of *P. roqueforti* and *P. paneum* is often seen in silage either in layers, on the surface or as lumps as big as 40 cm in diameter in the middle of stacks (Figure 1). The colour is green often in grey or blue shades and *P. roqueforti* and *P. paneum* cannot be differentiated visually on the silage.

Other very common fungi are various species of *Mucor* and *Rhizopus* (class Zygomycetes), which have been isolated from all types of silage [21–23*, 31, 32*–34, 40*]. They grow rapidly especially in partly aerated outer layers of silage. The rapid growth of these species may obscure the growth of other less vigorous species during cultivation and identification in the laboratory.

Aspergillus fumigatus has also been isolated from silages all over the world, both in warm [21, 41] and temperate [22, 23*, 33, 34, 40*] climates. It has a high temperature optimum and tolerates temperatures up to 55°C [42] and can therefore often be observed near degraded outer layers of silage stacks where the microbial heat from degradation has selected for heat-tolerant species.

Other species often encountered are *Monascus ruber* [23*, 33, 40*, 43] and *Byssochlamys nivea* [22, 23*, 44]. *M. ruber* often produces red pigments and can be seen as lumps both near surfaces and in central parts of silage stacks. *B. nivea* and the anamorphic form *Paecilomyces niveus* produce white colonies in silage. *B. nivea* can survive acidic and anaerobic conditions and the ascospores are heat-resistant, as illustrated by the fact that it is an important contaminant of canned fruit and fruit juices [42].

Fusarium spp. have been isolated from silage in several cases [21, 23*, 40*]. Fusaria are generally not capable of surviving the ensiling process. Only *F. oxysporum* is known to survive under acidic and anoxic conditions [42]. Mansfield and Kuldau [30*] registered several species of *Fusarium* in fresh maize but none after ensiling. The survival of spores or recolonisation after opening may explain findings of Fusaria in silage.

Classic mycological determination of mycobiota by dilution and plating may unfortunately not reflect the actual growth of filamentous fungi in field and silage. This is a classic mycological dilemma already mentioned in a review of silage mycology by Pelhate [31]. The use of suitable media and incubation in modified atmosphere may give a more representative picture of the actual mycobiota in silage, but standardised procedures need to be developed. Even so heavily sporulating species like *P. roqueforti* may be overestimated. Silage cannot be considered a homogenous medium either. Within a stack or bale there are many ecological niches. For instance *P. roqueforti* is often observed as layers at a depth of 20–80 cm [Storm IMLD, unpublished, 33] where the O_2 concentration is too low for most spoilage organisms. In the outer layers *P. roqueforti* has been out competed by yeasts, bacteria and other filamentous fungi. Molecular biological techniques can in theory reveal the presence of all fungi in

Table 2. The most common fungal postharvest contaminants of silage, some known secondary metabolites and secondary metabolites confirmed in silage.

Species	Secondary metabolites	Detected in silage[a]	Reference
Penicillium roqueforti[b]	Agroclavine	+	[8]
	Eremofortin C		
	Mycophenolic acid	+, 1.3, 35, 117	[2, 8, 34, Nielsen KF, unpublished]
	PR-toxin	+	[Nielsen KF, unpublished]
	PR-amide and PR-imine		
P. roqueforti	Roquefortine A, D, 16-OH-roquefortine	+	[8]
and *P. paneum*[b]	Roquefortine C	+, 5.7, 36, 50	[8, 35, 82, Nielsen KF, unpublished]
	Andrastin A, B and C	+	[8, Nielsen KF, unpublished]
	Citreoisocoumarin	+	[8]
	Orsellinic acid		
	Festuclavine	+	[8]
P. paneum[b]	Marcfortine A	+	[8]
	Marcfortine B and C		
	Patulin	1.2, 40	[44, 82]
	Gentisic acid		
Aspergillus fumigatus[c]	Gliotoxin	0.878	[23*]
	bis-dethio-bis(methylthio)-gliotoxin	+	[Nielsen KF, unpublished]
	Fumigatins		
	Trypacidins		
	Sphingofungins		
	Pseurotins		
	Helvolic Acid		
	Fumagillins		
	Fumigaclavines		
	Fumitremorgines		
	Diketopioperazines		
	Fumiquinazolines		
Byssochlamys nivea/	Patulin	1.2, 40	[44, 82]
Paecilomyces niveus	Byssochlamic acid		
	Mycophenolic acid	+, 1.3, 35, 117	[8, 34, 82, Nielsen KF, unpublished]
Monascus ruber	Citrinin	0.037, 0.064, 0.25	[23*, 40*, 43]
	Monacolins	65	[43]
	Pigments, eg, ankaflavin		
	Monascopyridines		
Zygomycetes	May cause zygomycosis especially in immunocompromised animals		[76]
Geotrichum candidum	May reduce palatability of silage		[31]

[a]+: Metabolite detected in silage samples. Numbers state maximum concentrations in mg/kg where quantitative determination has been performed.
[b]Based on [8, 103*]
[c]226 Extrolites registered by Frisvad *et al.* [75*]

silage. Mansfield and Kuldau [30*] compared a DNA-sequence based technique with plating on malt-yeast sucrose agar (MYSA) and Nash medium (NASH) and found a much greater abundance of species with the molecular technique. Again dormant spores can give misleading results and the quantity of DNA cannot be correlated with the amount of mycotoxins.

Mycotoxins and other secondary metabolites

The above mentioned fungi are known to produce a wide range of mycotoxins and other secondary metabolites. But the production of these is very substrate dependent and not all may be present in silage. The complex microbial ecosystem of silage can also account for degradation and binding of such compounds.

Preharvest

Of the *Fusarium* derived mycotoxins, the trichothecenes are sesquiterpenes and are produced by various species of *Fusarium*. The compounds are divided into type A and B trichothecenes. Type A trichothecenes (mainly diacetoxyscirpenol [DAS], T-2 toxin and deacetylated analogues of these) are mainly produced by *F. poae*, *F. sporotrichioides* and *F. langsethiae* and are considered more toxic than type B trichothecenes (mainly DON and nivalenol [NIV], fusarenone-X, 3- and 15-acetyl-DON as well as acetylated and deacetylated analogues of these), which are primarily produced by *F. cerealis*, *F. culmorum* and *F. graminearum* [45]. Trichothecenes have a variety of toxic effects like vomiting (DON), reduced feed uptake and immuno-suppression as the most pronounced [46]. DON is usually the predominant trichothecene in crops and is therefore also the best studied. ZEA and α- and β-zearalenol (α- and β-ZOL) are estrogenic compounds mainly produced by the trichothecene type B producing *Fusarium* species [45]. In a survey of mycotoxins in various Dutch silage types, DON and ZEA were almost completely absent in grass silage, while they were highly abundant in maize silage [47*], despite the absence of the producing organisms postharvest [30*].

Fumonisins are sphinganine analogues with carcinogenic properties [48] and are primarily produced by *F. proliferatum* and *F. verticillioides* [45]. These species are mainly present in tropical and subtropical areas and fumonisin contaminations of preharvest crops are therefore higher in these areas. There are several groups of fumonisins with several members, but fumonisin B_1 (FB1) is the predominant and best studied analogue.

DON and FB_1 were shown to be less stable than ZEA in a lab scale experiment with ensiled maize [49*]. The maximum toxin degradation observed for DON, FB_1 and ZEA was 100%, 92% and 53%, respectively [49*]. The experiments also showed that storage time and dry matter content are more important than temperature. In a study of fresh and ensiled maize, DON levels were reduced by 57% in 3–6 month old silage stacks [50]. These observations suggest a substantial degradation of DON during ensiling, which is a fate that the other trichothecenes are likely to share. Some removal of field produced mycotoxins can be attributed to lactic acid bacteria. *In vitro* studies suggest that binding of DON, ZEA and FB1 is the major mode of action for lactic acid bacteria [51].

Plants are able to reduce the toxicity of mycotoxins formed in the fields for example by conjugation of mycotoxins to polar substances such as sugars, amino acids or sulphate. Natural occurring glucoside conjugates of ZEA [52] and deoxynivenol [53] have been detected. The conjugated forms will not be detected by standard methods designed for the precursor mycotoxins as they may be harder to extract and have altered chromatography. This means that the actual amount of mycotoxins may be underestimated due to masked conjugated mycotoxins.

Species of *Fusarium* can produce several other types of mycotoxins in cereals and maize preharvest, including moniliformin, fusaproliferin, beauvericin and enniatins, but very little is known about their stability in silage. The predominant enniatin analogue, enniatin B, was detected at levels up to 218 ng/g in 3-month-old maize silage stacks, while the related beauvericin occurred less frequently and at levels up to 63 ng/g. Enniatin levels in 3, 7 and 11 month old silage were not different from each other but were all lower than in freshly harvested maize [54]. This suggests that some of the enniatins were degraded within the first 3 months. In another study of preharvest maize, moniliformin was only produced in insignificant low ppb levels [55].

The four most frequently occurring *Alternaria* species in cereals and maize are *Alt. arborescens, Alt. alternata, Alt. tenuissima* and *Alt. infectoria,* which are able to produce a wide range of compounds with disputed toxicity. *Alt. arborescens, Alt. alternata* and *Alt. tenuissima* can produce alternariols, altertoxins, altenuene and tenuazonic acid [56], but there are only few reports on the natural occurrence of these compounds in small grain cereals preharvest, summarised in [57]. *Alt. infectoria* can produce infectopyrones and novaezelandins [58], but their natural occurrence has not been studied. One paper [59] also reports finding the *Alternaria* mycotoxins AAL-toxin A and B in silage. Liquid chromatography–mass spectrometry (LC-MS) with only one SIM ion (not very specific in such dirty matrix) was used to substantiate this very interesting finding, and since only one isolate (tomato pathogen *Alt. arborescens,* syn. *Alt. alternata* f. sp. *lycopersici*) in the world until now has been found to produce AAL toxins, the findings of AAL toxins in silages seems unlikely and needs proper validation.

With *Aspergillus flavus* and *Asp. parasiticus* present in crops and silage, aflatoxins may be produced. These are the most important group of mycotoxin produced by this organism, and mainly includes the B_1, B_2, G_1 and G_2 analogues, which are all produced by *Asp. parasiticus*, whereas *Asp. flavus* can

only produce B_1 and B_2 [60]. Aflatoxins are the most carcinogenic of known secondary metabolites and their occurrence in silage can be of great concern to human health as they can be transformed by cattle to hydroxylated derivates (aflatoxins M_1 and M_2), which can be found in meat and milk products. Other mycotoxins from *A. flavus* are cyclopiazonic acid and 3-nitropropionic acids. Aflatoxin B_1 has been detected in silage in some surveys while others have looked for it with negative results (Table 3)

Postharvest

P. roqueforti and *P. paneum* are the most widespread species of filamentous fungi in silages and they have on several occasions been associated with ill-thrift and disease in cattle herds [8, 10, 11]. As seen in Table 2 they produce a wide range of secondary metabolites *in vitro* and many of them have also been detected in silage.

The roquefortines are very ubiquitous and have therefore been suspected to be involved in toxicoses [61]. Data on neurotoxicity [62] and antibiotic properties [63] are published but no acute toxicity and a low transfer to organs and tissue were observed in feeding experiments with sheep [64]. PR-toxin (only produced by *P. roqueforti*) on the other hand has acute toxic effects in rats and mice [62, 65] but its fate in ruminants is unknown. Another known toxin, patulin, is produced by *P. paneum* as well as *B. nivea*. Patulin damages the kidneys and the gastro-intestinal tract functions in rats [66] and may reduce male fertility [67]. It has antibiotic properties [68] and is immunosuppressive at high doses [69, 70]. It does however form adducts with S-containing amino acids [71, 72] and may therefore not be bio-available in ruminants. Another commonly encountered metabolite is mycophenolic acid, which is produced by both *P. roqueforti* and *B. nivea*. It is antibiotic and immunosuppressant [73, 74]. The andrastins and marcfortines have not been tested in higher animals. The clavines are similar to alkaloids produced by *Neotyphodium* endophytes in Fescue grass preharvest and may thus result in similar symptoms, however ergovaline is considered the most important toxin involved in Fescue toxicosis. In a recent survey by Driehuis *et al.* [47*] roquefortine C was reported only in 1 of 120 grass silages and none of 140 maize silages. Mycophenolic acid was not found in any samples. Sampling of the silages was however conducted only 1–2 months after harvest and the stacks were still completely sealed so growth of postharvest contaminants was unlikely.

The widespread presence of *Asp. fumigatus* in silage naturally calls for concern. It is a known producer of more than 200 secondary metabolites [75*], including the potent gliotoxin, and may cause invasive infections in animals (Aspergillosis) [76]. Many of the metabolites are known to have antimicrobial, antifungal or antiprotozoan effects [75*] and may thus affect the microbiota of the rumen. Others, like gliotoxin, are immunosuppressive [77]. Silage samples contaminated with *Asp. fumigatus* have been analysed for gliotoxin only, which is produced in highest amount on substrates with a low C/N ratio. Gliotoxin may therefore not be a very good marker for presence of *Asp. fumigatus* toxins in silage. In *Monascus ruber* infected silage, citrinin has been detected. Citrinin is nephrotoxic [78], while the monacolins produced by the same species have no toxic effects and are used as cholesterol-lowering drugs.

Some Zygomycetes can, via endophytic bacteria, produce several bioactive secondary metabolites [79, 80], but the distribution of toxigenic isolates is not well examined. The fast growth of Zygomycetes may spoil large amount of silage very rapidly. Furthermore some species are known to cause invasive infections, Zygomycosis [76], especially in immuno-compromised individuals.

Toxicology

Mycotoxins in silage can affect animal health and productivity [18**]. Exposure of humans via transfer of mycotoxins to food (eg, milk) is also of concern [2, 18**]. The mycotoxins contaminating silage can induce carcinogenic, estrogenic or immunosuppressive effects. Feed refusal, birth defects, kidney, liver or lung damages, etc have also been observed in clinical trials [17], but acute intoxications causing death are rare [81]. Animals feeding on silage may be exposed to a mixture of mycotoxins [23*, 40*, 47*, 82] and chronic exposure to low levels of mycotoxins may result in non-specific symptoms such as impaired immune system and increased infections or metabolic and hormonal imbalances [18**, 83]. The intoxication of animals under field conditions does not always match the concentration of specific toxins [18**]. A cocktail of toxins can give a stronger effect than the single toxins alone [78]. Furthermore, not all toxins in silage are described in literature since new secondary fungal metabolites are still discovered [84, 85].

A review of animal disease outbreaks due to *Fusarium* toxin contaminated feed has been given by Morgavi and Riley [83]. Clear signs of exposure to a specific toxin are rare under field conditions; for DON feed refusal has been reported in cattle, pigs and chickens. Fumonisins can induce brain lesions in horses - equine leucoencephalomalacia (ELEM) and lung damage in pigs - porcine pulmonary oedema syndrome (PPE) [83]. Mouldy maize silage infected with *P. roqueforti* produced loss of appetite, disturbance of rumen activity and gut inflammation in dairy cows [86]. Kristensen *et al.* [87] however did not see any significant effects on milk yield or rumen pH in a feeding experiment where cows were fed alternating rations, including a ration with DON-contaminated maize silage and one with *Penicillium* contaminated maize silage. There were a few changes in the ruminal fermentation pattern that were significant.

Ruminants are often less susceptible to intoxication than other animal species. For instance they show lower responsiveness to DON, ZEA and fumonisins than pigs do [88–90]. The rumen microbiota can inactivate and degrade some mycotoxins, but not all types. For example, ochratoxin A is extensively degraded to the less toxic ochratoxin α [91], whereas ZEA is metabolised to the even more potent α -ZOL

Table 3. Confirmed examples of maize silage contaminated with *Fusarium, Aspergillus* and *Alternaria* toxins.

Mycotoxin	Country	Concentration (µg/kg)[a]		Reference
		Mean	Range	
Deoxynivalenol	Argentina		30–870	[104]
	France	160		[23*]
	France	204		[23*]
	Germany	2,919	?–3,944	[105]
	The Netherlands	651	nd–3,142	[47*]
	USA	600	nd–3,700	[50]
15-Acetyldeoxynivalenol	Germany	59	?–127	[105]
	The Netherlands	45	nd–1,013	[47*]
Nivalenol	Germany	1,612	?–2,809	[105]
HT-2 toxin	Germany	18	?–26	[105]
Scirpentriol	Germany	25	nd–124	[105]
Monoacetylscirpentriol	Germany	20	nd–49	[105]
Zearalenone	Argentina		nd–350	[104]
	France		<20	[23*]
	Germany	432	?–1,790	[105]
	The Netherlands	92	nd–943	[47*]
α-Zearalenol	Germany	3	nd–15	[105]
β-Zearalenol	Germany	23	nd–116	[105]
Fumonisin B_1	Argentina		340–2,490	[104]
	The Netherlands	463	nd–26,200	[47*]
	USA	2,020	nd–10,100	[59]
	USA	590	nd–1,824	[106]
Fumonisin B_2	The Netherlands	130	nd–7,800	[47*]
	USA	980	nd–20,300	[59]
	USA	66	nd–276	[106]
Fumonisin B_3	USA	29	nd –161	[106]
Enniatin B	Denmark	73	nd–218	[54]
Enniatin B_1	Denmark	10	nd–48	[54]
Beauvericin	Denmark	8	nd–63	[54]
Aflatoxin B_1	Argentina		nd–176	[104]
	Italy		nd–<4	[107]
	Mexico		500–5,000	[108]
	Brazil	nd		[109]
	USA	nd		[110]

[a]nd: not detected.

[89]. FB1 largely passes the forestomach in ruminants [90]. Animals with impaired rumen fermentation are expected to metabolise toxins less effectively. Patulin is an example of a mycotoxin with antibacterial properties that can disturb the rumen fermentation [92]. Keese *et al.* [93] have also detected alterations in the ruminal fermentation pattern when cows were fed a ration containing 5.3 mg/kg DM of DON. High-yielding dairy cows may be more susceptible to diseases caused by mycotoxins, maybe due to a higher level of stress [94].

Milk can be contaminated with the carcinogenic metabolite aflatoxin M_1 [95], when lactating animals are exposed to the mycotoxin aflatoxin B_1 in feedstuffs. Up to 6% of the administered dose of aflatoxin is excreted in the milk [96]. Carry over rates of DON, ZEA, ochratoxin A, and fumonisins from feed to milk are much lower than aflatoxin. Hence humans are not significantly exposed to these four toxins through milk [88–91]. The carry-over rates from feed to milk of *P. paneum* and *P. roqueforti* toxins, eg, PR-toxin, roquefortines or festuclavine are not known [2].

Many countries have regulatory limits for mycotoxins in feed. Maximum acceptable levels of DON (0.9–12 mg/kg feed), ZEA (0.1–3 mg/kg), ochratoxin A (0.05– 0.25 mg/kg) and fumonisins (5–60 mg/kg) in feed material have been set by the European Union. These values are toxin, feed-type, and animal dependent, and address animal welfare, as the exposure of humans through animal products is low [97]. Maximum levels of aflatoxin B_1 (0.005–0.02 mg/kg) in feed is regulated based on human safety as it is a genotoxic carcinogen [98]. The lowest value in feed applies to dairy cattle due to carry-over in milk. As seen in Table 3 mycotoxin levels in silage rarely exceed the existing regulatory limits.

Preventive agricultural practices

In order to minimise the risk of fungal spoilage and mycotoxin contamination of silage, farmers can implement different strategic and practical approaches.

Preharvest infection of crops cannot be eliminated. Incidents and concentrations of preharvest toxins are very dependent on weather conditions, and models to predict the spread of plant pathogens have been developed [99]. In a survey by Mansfield *et al.* [50] agronomic practices had no effect on incidence of DON, but the concentrations were significantly higher in no till-systems than in mixed till and mouldboard till systems.

To avoid spoilage of silage in silos and bales there are several practical approaches to consider. Proper chopping, thorough compaction and sealing are very important factors for limiting the oxygen supply, which is of utmost importance. O'Brien *et al.* [32*] found that visible damage to the polythene film of baled grass silage was the only bale production and storage characteristic that significantly predisposed bales to increased fungal spoilage. Furthermore, a positive correlation was observed between polythene film damage and dry-matter content [100] most likely because dry and stiff stems are more likely to puncture the film. For silage in stacks and silos, the compaction is very important both for the quick achievement of anaerobic conditions and for minimisation of O_2 infiltration from the cutting front. Therefore particle size must not be too big as this hinders compaction. Special equipment for cutting silage rather than grabbing it from the stack may also minimise O_2 infiltration. Proportionating silage stacks to the rate of use may also help, as low rate of use has been associated with spoiled silage [33]. Optimal dry-matter content of the crop is also important for the initiation and course of the silage fermentation. Significant negative correlation between dry matter content and concentration of lactic, acetic, propionic and butyric acid was observed [32*].

In order to affect the fermentation process, silage additives can be added during silage making. These may be acids intended to restrict growth of undesirable organisms from the start, fermentable sugars (eg, molasses) to stimulate production of organic acids or biological inoculants to increase the concentration of desired microorganisms in silage. Biological additives are the most popular type worldwide but may be used in combination with the other types [1]. Biological inoculants are however not always successful and there are both advantages and disadvantages to them [101].

Conclusion

Silage can contain a wide range of mycotoxins and other secondary metabolites originating from preharvest infection of crops or from postharvest infection in silos, stacks and bales. This has been associated with ill-thrift and disease in cattle, but the evidence for acute intoxication caused by contaminated silage is rare. Many of the filamentous fungi associated with silage are however producers of antimicrobial and immunosuppressive compounds. It is possible that complex mixtures of these may result in sub-acute symptoms, ie, impaired rumen function or increased susceptibility to infections. This subject calls for further investigation.

The mycobiota of silage has been examined in several cases around the world, and the results are fairly consistent with *P. roqueforti* and *Asp. fumigatus* as some of the most abundant species. An often encountered group of filamentous fungi is the Zygomycetes but the possible effects of these have not been examined. The interplay between filamentous fungi, bacteria and yeasts is also an issue of interest, which may be able to explain the occurrence of filamentous fungi in the middle of otherwise well-preserved and managed silages.

Many of the secondary metabolites produced by known contaminants of silage have not been analysed for in silage. It is thus possible that there are so far undetected metabolites playing a role in intoxications with silage. The list of possible contaminants is very long and silage is an extremely difficult matrix since it is full of organic acids, sugars, chlorophyll and numerous other small molecules, of which many cannot

be easily removed by, eg, reversed phase solid phase extraction. Very few methods in silage have been published so there is a need for high specificity methods like LC-MS/MS with at least two transitions or daughter ion scans.

Acknowledgements

The authors wish to thank the Danish Directorate for Food, Fisheries and Agri Business (grant FFS05-3), the Danish Cattle Federation and the research school FOOD for funding.

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Original paper II

Storm, I.M.L.D., Kristensen, N.B., Raun, B.M.L., Smedsgaard, J., and Thrane, U. Dynamics in the microbiology of maize silage during whole-season storage. *Submitted to Microbial Ecology*.

Dynamics in the microbiology of maize silage during whole-season storage

Ida M.L.Drejer Storm[a]*, Niels B. Kristensen[b], Birgitte L. Raun[b], Jørn Smedsgaard[c], Ulf Thrane[a]

[a] Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, Kgs. Lyngby, Denmark.

[b]Department of Animal Health and Bioscience, Faculty of Agricultural Sciences, Aarhus University, Tjele, Denmark.

[c]The National Food Institute, Technical University of Denmark, Søborg, Denmark

*Corresponding author address:

Center for Microbial Biotechnology

Technical University of Denmark

Søltofts Plads, Building 221

DK-2800 Kgs. Lyngby

Denmark

E-mail: id@bio.dtu.dk

Phone: +45 4525 2608

Fax: +45 4588 4148

Abstract

Maize silage is an increasingly important feed product in cattle and dairy production. In modern production systems it is often stored for 14 months or more. Unfortunately maize silages are regularly spoiled by filamentous fungi and yeasts during storage, which may lead to mycotoxin contamination and subsequent health risks for animals, farmers and possibly dairy and meat consumers.

To monitor seasonal variations in the microbiology of maize silage and determine if the risk of fungal spoilage varies over the season, a continuous survey of 20 maize silage stacks was conducted over a period from three to 11 months after ensiling. The numbers of colony forming units (CFU) of filamentous fungi, yeasts and lactic acid bacteria were assessed at each sampling time-point and the cultivable species of filamentous fungi in both healthy looking and visibly mouldy samples were isolated and identified.

Significant differences in the number of CFU of filamentous fungi, yeast and lactic acid bacteria were detected over the eight months period of the experiment. The highest CFU of fungi was in March and May, five to seven months after ensiling, with average $\log_{10}$CFU values of 3.2, 3.3 and 6.8 for filamentous fungi on V8 and DG18 and yeasts on MYGP, respectively. The lowest numbers were detected in September, 11 months after ensiling, with averages of 0.7, 0.7 and 4.9 $\log_{10}$CFU, respectively. Filamentous fungi were isolated from all stacks at all time-points. The most abundant toxigenic mould species were *Penicillium roqueforti*, *P. paneum* and *Aspergillus fumigatus*. Their occurrence followed the same variation over the season and peaked in March and May, where the three species were isolated from 100, 65, and 60% of the samples silage stacks. The occurrence of *Zygomycetes* peaked in July where they could be isolated from 80% of stacks.

42 It was concluded that there are significant variations in the microbiology of maize silage over a
43 whole storage season. The risk of fungal spoilage was highest 5-7 months after ensiling and
44 lowest after 11 months. This information may be useful for the sampling of silage for post-
45 harvest mycotoxin contamination and in the management of maize silage stacks, when whole-
46 season storage is needed. Fungal spoilage and subsequent contamination with mycotoxins can
47 possibly be minimised by keeping silage stacks closed for longer periods of time before opening.

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Introduction

Maize silage is a widely used feed product for cattle. In many countries around the world the production of maize silage is equal to or larger than the production of grass silage [53] and in Denmark the production of maize for silage has increased more than 700% from 1990 to 2008 [3]. In North America and Europe cattle farming is increasingly based on free stall housing systems where a stable total mixed ration is fed all year round [52], so silages are in many cases stored for 14 months or longer. Therefore more information on the microbiology of maize silage during whole-season storage is needed.

Maize silage is produced by a lactic acid fermentation of whole chopped maize plants. Chopped plant material is compacted in large stacks or clamp silos, covered with plastic and left to ferment. Sometimes chemical or microbial additives are used to control the fermentation, but mostly the process relies on the naturally occurring lactic acid bacteria (LAB) from the plants. The initial enzymatic activity and following microbial activity depletes oxygen and produces carbon dioxide and organic acids, primarily lactic and acetic acid. The result is silage with 0-2% oxygen, 15-90% carbon dioxide and a pH around 3.8 [12, 51]. The biochemistry of silage is thoroughly reviewed by McDonald [22].

The microbial ecosystem of silage is dominated by LAB with the homofermentative *Lactobacillus plantarum* being the most frequently isolated from silage in general [22], but heterofermentative species e.g. *L. buchneri* and *L. brevis* are common [19, 39, 45] . Very often yeasts and filamentous fungi are also present in silage. Filamentous fungi often occur in silage as fist or ball sized lumps or as layers 20-50 cm from the surface [4, 27]. As growth of fungi in most cases is associated with production of mycotoxins, this can lead to mycotoxin

72 contamination of the silage [47] and hence be harmful to cattle, farm workers and dairy product
73 consumers [11] .

74 The dynamics of the microbiology of silage of different crops has been studied extensively for
75 the first days, weeks and months after ensiling [22, 23, 25, 26] giving a good understanding of
76 the principles of ensiling. However, to our knowledge only very few examinations of silage
77 mycobiota include silages more than six months old [15, 35, 37] and none of those are surveys.
78 With the changes in agricultural practices towards all-year feeding of silages, silages are often 14
79 months or more old at the time of feeding. A better understanding of the long-term dynamics of
80 silage is therefore important to optimize long term storage, minimize fungal deterioration and
81 decrease the risk of mycotoxins in silages.

82 The goals of this study are to monitor the seasonal variations in the microbiology of maize silage
83 and determine whether the risk of fungal spoilage and contamination of maize silage *in situ*
84 varies over a whole season. This was done by a) determining the fungal species present in maize
85 silage stacks used for feed-out at selected farms from three to 11 months after ensiling, b)
86 determining whether the number of viable microorganisms in silages varied during this period
87 and c) examining whether microbial variations between stacks and over time correlated with
88 physical and chemical parameters of the silage.

89 **Materials and methods**

90 *Sample collection*

91 20 dairy farms within two regions of Denmark were randomly selected among farms in the
92 Danish Cattle Federation database (Danish Cattle Federation, Aarhus, Denmark) which were
93 expected to feed maize silage throughout the season. Ensilage had been performed between 1st

September and 20th October 2006.All farms were visited five times with two month intervals from January to September 2007. The samples were collected as described by [34]. The procedures of relevance for this study are described below.

At each visit silage samples were obtained by drilling vertical cores with an automated steel borer (Ø=50 mm; Frøsalget, Brørup, Denmark) approximately one meter behind the bunker face of the stacks. Protective nets and covering were retracted and samples were taken in full depth of the stack, except the 0.3 m nearest the bottom, to avoid damage to the drill. In the case of very low silage stacks two or three cores were taken in the same area and combined. Each primary sample (2-3 kg) was collected in a large plastic bucket and mixed thoroughly by hand before taking out secondary samples of 500-800 g. Samples were double-bagged and air excluded by hand. Silage height, centre temperature 15 cm behind the face of silage, variety of maize and use of silage additives was also noted at each visit.

Samples of spoiled silage with visible fungal growth (hot-spots) were also sampled. In cases with many similar hot-spots a few representative samples were collected. Hot-spots were sealed in individual plastic bags and their position in the stack was noted.

Culturing and fungal identification

All samples for microbial analysis were stored cold until sample reduction and analysis was performed. Samples were kept cold by freezing elements from sampling until arrival at the laboratory where they were stored at 0-3°C. All sampling was done aseptically using sterile gloves and utensils cleaned with 70% ethanol: Samples were mixed well by hand, laid out in an oblong pile and divided by moving equal slices of the pile to two alternate bags. If necessary the procedure was repeated with one of the reduced samples until a sample size of approximately

200 g was reached. This sample was mixed well by hand and distributed in a 2-3 cm thick layer on a sterile plastic bag covering a steel tray marked with a 5x5 cm grid. The distribution was performed one thin layer at a time. Finally silage from one randomly chosen square of the marked grid at a time was transferred to sterile plastic-bags until the desired sample sizes of 40 g for serial dilution and 20 g for direct plating was reached.

The 40 g for serial dilution was mixed with 360 g of sterile 0.1% peptone water and homogenized for 2 min, medium speed in a Stomacher laboratory blender (Seward Medical, London, UK). Serial dilutions were performed with 0.1% peptone water and inoculated by spread plate technique according to [41].

Lactic acid bacteria were enumerated on Mann, Rogosa, Sharp (MRS) medium (Oxoid) containing 50 mg/L nystatin. Nystatin was added as a concentrated aseptic solution of the compound in 70% ethanol just prior to pouring of the plates. Plates were incubated for three days at 25°C under anaerobic conditions. 0.1 ml of the dilutions, corresponding to 10^{-4} – 10^{-9} g silage per dish, were plated. Yeasts were enumerated on malt yeast glucose peptone plates (MYGP) with antibiotics after 4 days incubation at 25°C. The medium contained 10 g·L^{-1} D(+)-glucose, 5 g·L^{-1} Bacto peptone, 3 g·L^{-1} yeast extract, 3 g·L^{-1} Bacto malt extract, 20 g·L^{-1} agar, 50 mg·L^{-1} chloramphenicol, and 50 mg·L^{-1} chlorotetracylin. Dilutions corresponding to 10^{-2} – 10^{-8} g silage were plated. Filamentous fungi were enumerated on V8-juice agar plates (V8) and dichloran glycerol 18% agar plates (DG18) [41] both containing 50 mg·L^{-1} chloramphenicol and chlorotetracyclin. Dilutions corresponding to 10^{-2} – 10^{-7} g silage were plated and enumeration performed after 7 days incubation at 25°C. Duplicate plating of each dilution series was performed on all media. The number of CFUs on each plate was recorded when it was below 400, 400, and 100 for LAB, yeasts and filamentous fungi, respectively. For each sample on each

140 medium the CFU was calculated as the sum of colonies of all plates divided by the total amount
141 of silage plated.
142 For qualitative determination of low concentration mould species 20 g silage was evenly
143 distributed on 5 Petri dishes of V8 and 5 of DG18 under aseptic conditions. From the collected
144 hot-spots one streak with an inoculation needle and one piece of clearly infected silage was both
145 inoculated on one Petri dish V8 and one dish DG18. Qualitative plates were incubated for 6-7
146 days at 25^{o}C.

147 Identification of fungal species was performed according to [41] and species confirmed by Jens
148 Chr. Frisvad, Birgitte Andersen and Ulf Thrane, Technical University of Denmark.

149 *Physical and chemical analysis*

150 In the collected silage samples dry matter content, pH, ethanol, propanol, 2-butanol, propanal,
151 ethyl acetate, propyl acetate, propylene glycol, D-glucose, L-lactate, ammonia, acetate,
152 propionate, and butyrate were determined as described by [34].

153 *Mycotoxin analysis*

154 Freeze-dried portions of the drilled silage samples from all 20 farms collected in January were
155 analysed for concentration of the *Fusarium* toxins: Deoxynivalenol (DON), 3-acetyl DON, T-2,
156 HT-2 and fuseranon X at The Danish Plant Directorate employing their method for
157 "Trichothecenes in feed". The method applies to determinations of several trichothecenes and is
158 presently accredited for DON, HT-2 and T-2. Briefly, silage is extracted with acetonitril:water
159 (84:16 v/v) and filtered extracts cleaned up with Mycosep® 227 columns (RomerLabs, Tulln,
160 Austria). Samples are spiked with C^{13}-labeled internal standards and analysed by HPLC-triple
161 quadropole mass spectrometry.

Statistical analysis

All microbial counts of CFU were logarithmically transformed (base-10) to logCFU prior to statistical analysis. Calculations were performed with either limit of quantification set to 1 $CFU \cdot g^{-1}$, under the assumption that silage is never free from fungal propagules, or with these values as missing values.

Compliance with at normal distribution was assessed by Shapiro-Wilk, Kolmogorov-Smirnov, Anderson-Darling and Cramér-von Mises goodness-of-fit tests in the PROC UNIVARIAT procedure of SAS [1] and by visual inspection of histograms and normal probability plots. Variance homogeneity was checked by plotting standardized residuals against predicted values and against all variables in the model.

Effect of time was evaluated by analysis of variance using the PROC MIXED procedure of SAS 9.1 [1] on a model describing logCFU as a function of time and the random variable farm. The initial model considered time as repeated measurement on farm. The significance of variance components in the model was tested with a restricted/residual likelihood ratio test, comparing the difference in negative restricted/residual log-likelihood values of the reduced and the full model to a χ^2_{df}-distribution with df equal to the number of parameters eliminated in the reduced model. A significance level of 0.05 was applied.

Data was checked for cross-correlations between the microbial and physical/chemical data using The Unscrambler 9.2 [2] by examining 2-by-2 cross-correlations and performing partial least squares regression (PLS). All variables were scaled by their standard deviation and the model assessed by full cross validation [2].

Results

During the 8 months sampling period from 3 to 11 months after ensilage there were significant changes in the number of CFUs of filamentous fungi found on V8 and DG18, of yeast and of LAB (P=0.0005, P=0.0003, P<0.0001 and P=0.0016, respectively). Log_{10}CFU of filamentous fungi and yeast were highest in spring 5 to 7 months after ensiling and lowest in September 11 months after ensiling (Figure 1). Fewer hot-spots were also observed and collected in September.

Filamentous fungi were isolated from all farms at all sampling times. The most commonly isolated filamentous fungi were *Penicillium roqueforti*, species of *Zygomycetes* (primarily *Mucor* sp.), *P. paneum*, and *Aspergillus fumigatus* (Table 1). The same species also dominated in the collected hot-spots of fungal growth (Table 2). Less frequent species were *Geotrichum candidum*, *Byssochlamys nivea*, *Coelomycetes*, *Monascus ruber* and species of *Penicillium* (other than *P. roqueforti* and *P. paneum*). Other species rarely encountered included *Trichoderma* sp., *Eurotium* sp., *Fusarium* sp., one *Cladosporium* sp., one *Aspergillus flavus* and one *A. niger*. Other species also includes isolates which could not be identified due to lack of sporulation. They accounted for 9.6% of the total number of isolates and were most abundant in the July and September samples.

The occurrence of *P. roqueforti*, *P.paneum* and *A. fumigatus* followed the same trend as the overall CFU of filamentous fungi and peaked in the March and May samples. *Mucor* species and the other *Zygomycetes* were less frequent than the previous mentioned species until July, where they became the most frequently isolated group as well as the most frequent contaminants of hot-spots, and in September where they dominated the mycobiota of the collected hot-spots.

204 Very few *Fusarium* spp. were isolated but DON was present in all 20 samples from January with
205 an average concentration of 1056 µg·kg^{-1} (Table 3). T-2, HT-2 and fusarenone-X was present in
206 some of the analysed samples but 3-acetyl DON was not detected in any.

207 The tests of the statistical models showed that for some parameters models with a spatial
208 Gaussian or autoregressive covariance structure were better than the basic random farm model
209 (P=0.01 to 0.05), but regardless of the different covariance models, significance levels of the
210 factor time were all in the same range, and the simplest model with farm as a random variable
211 was used. Statistical analysis was also performed with a data set where CFU values below the
212 limit of quantification was counted as missing values, leading to the same overall conclusions on
213 the effect of time ($P \leq 0.0022$ for all microorganisms).

214 No apparent connection between the occurrence of filamentous fungi and any of the other
215 microbial data or physical/chemical data was seen, except for correlation between the counts of
216 filamentous fungi on V8 and DG18. PLS regression between yeast counts (logMYGP) and the
217 physical/chemical parameters [34] gave a positive correlation between the yeast count and the
218 concentrations of ethanol and glucose and negative correlation with ammonia and temperature,
219 but with the optimal number of two principal components the model was not capable of
220 explaining more than 49 and 40% of the variation in the Y and X data, respectively. For the
221 counts of LAB, filamentous fungi on V8 and DG18 the percentages of explained Y-variance
222 were 45, 22 and 17%, respectively.

223 **Discussion**

224 This survey includes 20 farms from two regions of Denmark. Both field stacks and planar silos,
225 with and without concrete walls, different maize varieties, varying harvest times and uses of

226 additives were represented. As such the chosen farms can be considered as representative of the
227 majority of maize silage dependent dairy farms in Denmark. The survey only covers the year
228 2007, sampling silage grown in 2006. Year-to year variations can be expected as a consequence
229 of variations in growth and harvest conditions.

230 The analysis of the seasonal variations in microbial counts revealed significant differences over
231 the course of a storage season. Initially the counts of filamentous fungi increases but after 5-7
232 months of storage the amount of viable fungal propagules decreased. This may be due to a
233 reduction in the total number of fungal propagules or due to reduced viability of the propagules
234 after more than 7 months in the silage. 3 months of airtight storage has been found to decrease
235 the germinability of P. roqueforti spores [38]. Middelhoven and van Baalen [25] also found
236 anaerobic silage to be a hostile environment to yeast as the yeast counts peaked during the first
237 14 days and then gradually decreased over a period of 4 months. A lower number of yeast and
238 moulds should reduce the risk of spoilage upon aeration, as yeasts are believed to be the
239 microorganisms who initiate aerobic spoilage [20, 22, 24]. The environment in the silage stack
240 not only protects the silage when it prevails but also reduces the risk of spoilage upon aeration.

241 The number of collected hot-spots was also lower in September, which could be connected with
242 the lower number of viable fungal propagules. If the hot-spots only develop as the bunker face
243 approaches and atmospheric air diffuses into the stack, a lower number of fungal CFUs in the old
244 silage will lead to fewer hot-spots. The rate of use and stack management has also previously
245 been related to fungal spoilage, aerobic stability and loss of dry matter [27, 32, 40].

246 The possibility of seasonal temperature variations being the cause of the microbial variations
247 instead of storage time was also examined. There was no correlation between temperatures

248 measured 15 cm behind the bunker face at sampling [34] and any of the microbial parameters.
249 There was significant difference in silage temperature between sampling times [34]. Silage
250 temperatures were lowest in January and highest in July, following the same trend as air
251 temperatures (Figure 2). Silage temperatures were higher than air temperatures, especially
252 during winter, which can be explained by the microbial activity of the silage.

253 The identified species of filamentous fungi are in accordance with a review of publications on
254 the subject [47]. *P. roqueforti* and *P. paneum* were the dominant species and either or both were
255 isolated from 96% of the collected samples. The two species can be very difficult to separate in
256 culture as they are very similar and both sporulate heavily. In our experience *P. roqueforti* spores
257 are slightly more hydrophobic and spread more easily so the presence of *P. paneum* may be
258 overlooked. Both species are tolerant to acidic, low oxygen and high carbon dioxide
259 environments [14, 48, 50]. They are also capable of growing at temperatures of 5 to 10^{o}C [14,
260 36] which may explain their abundance early in the year, where stack temperatures at the bunker
261 faces averaged 13^{o}C. *P.roqueforti* and *P. paneum* are capable of producing both toxic,
262 immunosuppressive, antibacterial and other secondary metabolites with unknown toxicological
263 effects, including PR-toxin, patulin, roquefortine A-C, marcfortine A-C, andrastine, and
264 mycophenolic acid and many of these have been detected in silages [47]. The third member of
265 the *P. roqueforti* complex [6], *P. carneum*, was not observed, which is in accordance with [7].

266 The *Zygomycetes* are common saprobic fungi with world-wide occurrence in e.g. soil and
267 decaying plant material [41]. They are not known to be particularly resistant to the hostile silage
268 environment, but they sporulate heavily and furthermore grow very rapidly [41]. It is therefore
269 most likely that they out-compete or overgrow other more pH- and low O_2- tolerant fungi, once
270 the atmospheric conditions and pH is sufficiently modulated. In cultures they may completely

dominate other fungi, especially on V8. Even in cases where another species was obviously present it was impossible to isolate it and only tentative identifications were possible. The abundant detection of *Mucor* spp. can be related to the temperature level as *Mucor* is the generus within the *Zygomycetes* with the lowest temperature for optimal growth [41]. In general, *Zygomycetes* are not known to produce toxins themselves but can via endophytic bacteria produce bioactive secondary metabolites [16, 42]. *Zygomycetes* can also cause invasive fungal infections in man and animals, particularly in immuno-compromised individuals [8, 17].

A. fumigatus is often emphasized in mycological surveys of silage due to its production of many bioactive secondary metabolites [13] and the risk of infections in lungs and other tissue (Aspergillosis) [17, 18]. The percentage of stacks infected with *A. fumigatus* in the present survey ranged from 11-60 %, peaking in May after 7-8 months of ensiling. In literature, the incidence of *A. fumigatus* in silage is very variable ranging from 8-9 % of samples [27, 43] to 69-75 % of samples [9, 10]. This places the data of the present survey in the same range, indicating that the differences may be due to different times of sampling or possibly climatic variations. *A. fumigatus* colonies on the initial plates of V8 and DG18 were not dense and easily distinguishable and they often co-occurred with other species requiring experience to spot and separate them. The highly immunosuppressant and toxic metabolite gliotoxin has been detected in silages and on feed substrates [5, 30, 37] but the general levels of gliotoxin as well as the possible presence of other of the toxic *A. fumigatus* metabolites in silages still need to be elucidated.

Only very few Fusaria were isolated in the present study. *Fusarium* is however widespread in maize pre-harvest [33] and therefore *Fusarium* mycotoxins may be present after ensiling, which was also the case in the January samples of this study. In Denmark the concentrations of

deoxynivalenol, zearalenone and fumonisin $B_1 + B_2$ are low and only in a few cases has deoxynivalenol concentrations exceeded the regulatory limit of 8000 $\mu g \cdot kg^{-1}$ for cattle feed set by the European Commission (Jens L. Sørensen (2009) Preharvest fungi and their mycotoxins in maize. PhD thesis, Technical University of Denmark, Kgs. Lyngby, Denmark). The maximum concentration of DON detected in the present study was also well below the ECC limit. As very few Fusaria were isolated over the whole season the risk of post-harvest contamination with *Fusarium* toxins is very small. There are reports of *Fusarium* spp. in silage [9, 15, 30, 31, 37] while others do not or only in small numbers find different species of *Fusarium* [10, 21, 27, 29, 44]. Fusaria are generally not capable of growing under the conditions of well managed silage, except for *F. oxysporum* which can grow under anaerobic conditions [41, 49]. Insufficient ensiling and improper management may allow the growth of other *Fusarium* spp., explaining the above mentioned findings, and the use of *Fusarium* selective isolation procedures may increase the incidence of isolation.

The purpose of taking silage samples approximately one meter behind the bunker face was to represent the silage fed to livestock at different times of the year. As a consequence it was not necessarily the same stack which was sampled at each farm every time. Some farms had one large silage stack while others had several smaller stacks, so up to three different stacks were sampled at one farm during the course of the survey. However, all stacks at the same farm were considered reasonably identical and therefore the 20 farms can be considered as a randomly selected subsample of a larger group of farms with repeated measurement at each farm. Inspection of the raw data shows that there is a large variation both across farms and across time points, especially for filamentous fungi and yeast, which complies with a separate examination of the microbial heterogeneity of maize silage [46].

317 The $\log_{10}$ transformed CFU values complied with a normal distribution but there were some
318 differences in the variance between sampling time-points. As the statistical analysis assumes
319 variance homogeneity, the P-values may be underestimated, but with the clearly significant
320 effect of time, this should not affect the conclusions.

321 The lack of correlation between fungal logCFUs and physical/chemical properties of the silage
322 samples is probably due to a not measured factor which has a much larger impact on the
323 mycobiota of the silage samples. The availability of oxygen is a very important factor for fungal
324 growth and could have such a large impact on the fungal logCFUs. In this experiment the
325 samples were taken approximately one meter behind the cutting front. It is therefore possible that
326 the sampled silage is affected by intruding atmospheric air [20, 32, 51] which may have over-
327 shadowed other factors.

328 O'Brien et al [28] detected significant correlations between several of the physical and chemical
329 properties of baled grass silage, but no significant correlations with the proportions of visibly
330 fungal contaminated surface area. The clearly significant correlations between in particular DM
331 content and the chemical composition must be seen in the light of a much larger variation in
332 these variables in the baled grass silage than in the maize silages of this survey.

333 Based on this study we can conclude that long storage time of maize silage is associated with
334 significant changes in the microbiota of the silage. Filamentous fungi were ubiquitously present
335 in the sampled maize silages but the number of cultivable fungal propagules was highest 5-7
336 months after ensilage and lowest after 11 months. Thereby the risk of fungal spoilage upon
337 aeration is higher after 5-7 months than after 11 months of storage. There were no apparent

338 correlations between the number of cultivable microorganisms and the temperature or chemical
339 composition of the examined maize silages.

340 **Acknowledgements**

341 We wish to thank Rudolf Thøgersen (Danish Cattle Federation, Skejby, Denmark) for
342 establishing contact to the farmers and the twenty dairy farmers for allowing us to sample their
343 silage and providing background information. Anita Iversen and Niels Ellermann at the Danish
344 Plant Directorate, Kgs. Lyngby, Denmark are thanked for performing mycotoxin analysis. The
345 technical assistance of Jesper M. Mogensen at the Technical University of Denmark and of
346 Torkild N. Jakobsen, Ole H. Olsen and Birgit H. Løth at Faculty of Agricultural Sciences,
347 Aarhus University, is gratefully acknowledged. Funding was provided by The Directorate for
348 Food, Fisheries, and Agri Business (Copenhagen, Denmark (#FFS05-DJF-3)), the Faculty of
349 Agricultural Sciences at Aarhus University, Danish Cattle Federation, the Research School
350 FOOD, and the Technical University of Denmark.
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485 **Figure legends**

486 **Figure 1:** Averages of $\log_{10}$ transformed CFUs of filamentous fungi on V8 (▼) and DG18(♦),
487 lactic acid bacteria (■) and yeasts (●) from 20 maize silage stacks sampled approximately 3, 5, 7,
488 9 and 11 months after ensiling. Error bars indicate 95% confidence intervals.

489 **Figure 2:** Averages of temperatures measured 15 cm behind the silage bunker faces in 20 maize
490 silage stacks sampled approximately 3, 5, 7, 9 and 11 months after ensiling (■, error bars indicate
491 95% confidence intervals of the mean) [34]. For comparison the daily average air temperatures
492 from Skrydstrup, Denmark are included (–, Data provided by the Danish Meteorological
493 Institute, Copenhagen, Denmark).

494

Table 1: Number of sampled maize silage stacks from which specific species or groups of filamentous fungi have been isolated at the given sampling time.

	Month				
	Jan (n=20)	Mar (n=20)	May (n=20)	Jul (n=20)	Sep (n=18)
Penicillium roqueforti	17	20	18	15	12
Zygomycetes	7	15	14	16	9
Penicillium paneum	11	13	8	10	5
Aspergillus fumigatus	3	7	12	4	2
Geotrichum candidum	2	8	4	5	4
Byssochlamys nivea	4	3	2	5	4
Coelomycetes	2	4	4	3	3
Penicillium sp., other	0	4	0	0	4
Monascus ruber	5	1	0	0	1
Others	10	4	3	13	13

499 **Table 2:** Number of sampled maize silage stacks containing visible fungal hot-spots with
500 specific species or groups of filamentous fungi as primary contaminant, displayed by sampling
501 time.

	Month				
	Jan (n=20)	Mar (n=20)	May (n=20)	Jul (n=20)	Sep (n=18)
Penicillium roqueforti	9	10	11	5	2
Zygomycetes	5	7	10	14	7
Penicillium paneum	7	5	3	2	2
Aspergillus fumigatus	2	6	6	3	1
Geotrichum candidum	0	3	1	1	1
Byssochlamys nivea	3	0	2	3	1
Coelomycetes	2	0	3	2	0
Penicillium sp., other	0	0	0	1	1
Monascus ruber	4	0	0	0	1
Others	1	2	4	5	1
Total	33	33	40	35	17

502

503

504 **Table 3:** Summary statistics on the concentrations of *Fusarium* toxins detected in 20 maize

505 silage samples collected in January, approximately 3 months after ensilage.

Mycotoxin	n (positive samples)	Mean ($\mu g \cdot kg^{-1}$)	Min ($\mu g \cdot kg^{-1}$)	Max ($\mu g \cdot kg^{-1}$)
DON	20	1056	160	5094
T-2	1	2	0	2
HT-2	12	104	2	327
Fuseranon X	4	10	8	14

506

507

Figure 1

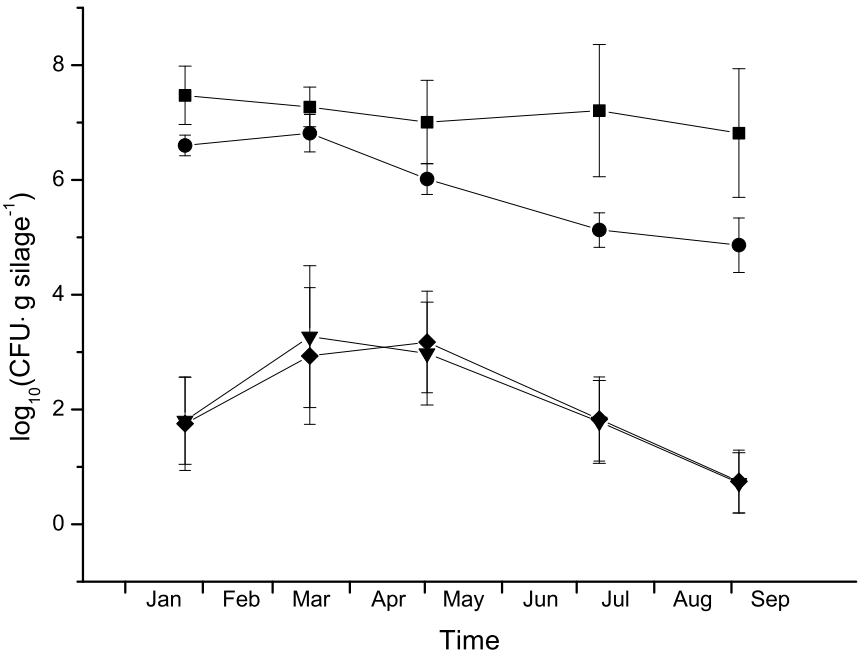
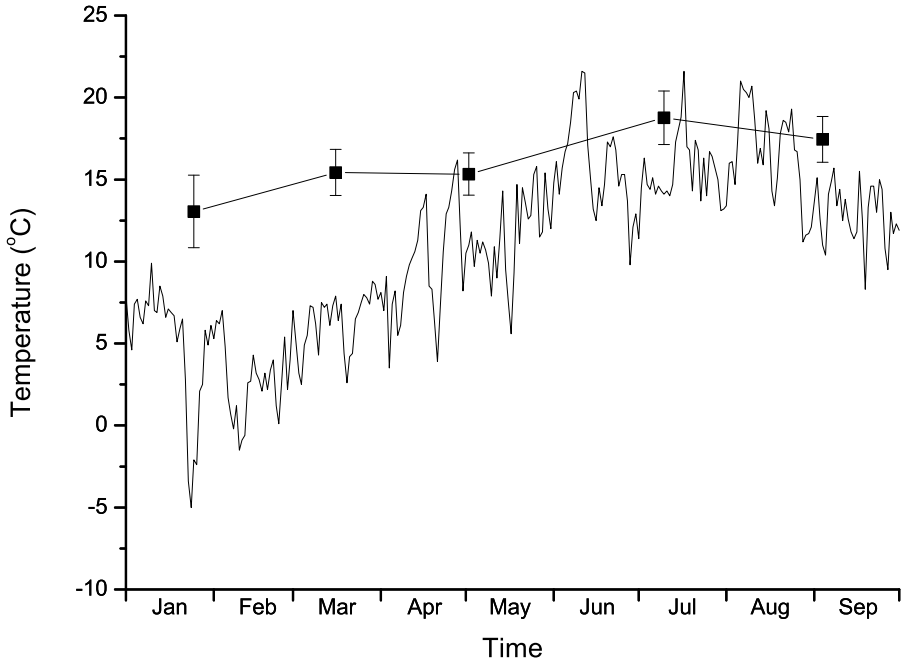


Figure 2



Original paper III

Storm, I.M.L.D., Thøgersen, R., Smedsgaard, J., and Thrane, U. Intra-stack heterogeneity of microbial and feed value parameters of maize silage. *Submitted to Animal Feed Science and Technology.*

Intra-stack heterogeneity of microbial and feed value parameters of maize silage

Ida MLD Storm[a]*, Rudolf Thøgersen[b], Jørn Smedsgaard[c], Ulf Thrane[a]

[a] Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark

[b] Danish Cattle Federation, Danish Agricultural Advisory Service, Århus, Denmark

[c]The National Food Institute, Technical University of Denmark, Søborg, Denmark

*Corresponding author address:

Center for Microbial Biotechnology

Technical University of Denmark

Søltofts Plads, Building 221

DK-2800 Kgs. Lyngby

Denmark

E-mail: id@bio.dtu.dk

Phone: +45 4525 2608

Fax: +45 4588 4148

Abstract

Maize silage stacks contain hundreds of tons of silage and therefore, by nature, pose a sampling problem as only a few kilos of silage can be sampled for determination of microbial properties and feed value. In general, to use results from these small sub-samples as the basis of conclusion for the entire silage stack requires either that the analytes are homogenously distributed in the stack or that a very large number of sub-samples are analysed.

Repeated sampling was conducted in 5 maize silage stacks, to evaluate the magnitude of sampling error compared to analytical errors from the methods of analysis. The collected samples were analysed for the contents of colony forming units of filamentous fungi, yeast, lactic acid bacteria, and feed value parameters.

All microbial parameters showed a significant variation between samples from the same stack. In particular the fungi were distributed highly heterogeneously with relative standard deviations between samples from the same stack of 36 % for filamentous fungi on V8, 39% for filamentous fungi on DG18, and 20% for yeast on MYGP. Lactic acid bacteria were more homogenously distributed with a relative standard deviation of 4% for samples from the same stack.

The feed value parameters had intra-stack relative standard deviations ranging from 1 to 11%. In this study 3 to 7 primary samples were needed to reach acceptable 95% confidence intervals for the mean values of the analysed feed value parameters.

The study demonstrates that variations between the number of colony forming units and feed value parameters in samples from the same silage stack are large. Values based on one or a few full depth samples from a whole silage stack must be interpreted with great care, as the error margins of such values are large. General conclusion about the microbial state or feed value of a whole maize silage stack should not be made on the basis of such a limited sample ratio. An alternative or supplemental

solution may therefore be regular analysis of samples from the freshly exposed cutting front of silage stacks.

Keywords: maize silage, heterogeneity, variance, sampling

Abbreviations:

LAB: lactic acid bacteria
CFU: colony forming units
V8: V8-juice agar
DG18: dichloran glycerol 18% agar
MYGP: malt yeast glucose peptone agar
MRS: Mann, Rogosa, Sharp agar
RSD: relative standard deviation
RMSEP: Root mean square error of prediction

Introduction

Over the last decades maize silage has become a very important feed component for cattle with the availability of short season maize varieties suitable for temperate climates (Wilkinson and Toivonen, 2003). In Denmark the production has increased by more than 700% from 1990 to 2008 (Statistics Denmark, 2009).

The production of maize silage is carried out on each farm with subsequent variations in e.g. cultivar, stage of growth, climate, fertilization, dry-matter content, chop length, stack or silo type, cover type, degree of compaction and use of silage additives. These differences lead to microbial and nutritional variations in the silage (McDonald et al., 1991). To optimise the feed rations and assess the microbial state of individual silage stacks, samples are routinely taken from stacks and analysed for physical, chemical and microbial properties. Decisions on composition of the feed ration and the use of silage additives are based on such analysis.

Silage stacks represents the classical sampling problem: They contain hundreds of tons of silage but only a few kilogram of silage can be sampled for analysis. Furthermore, the final analysis may only require a few grams of silage. Therefore it is crucial that sampling from silage stacks is done carefully, taking the unavoidable inhomogeneity of the silage stack into account to ensure that the results obtained are representative of a larger volume of the silage. The science of representative sampling has been studied extensively for e.g. soil sampling in geology (François-Bongarçon, 2004) or sampling for mycotoxins in food and feed (Whitaker et al., 2005). From these well examined areas it is known that the heterogeneity of a system and appropriate sampling techniques are dependent on both properties of the bulk product and of the contaminant (Whitaker et al., 2005). It is also well known that the variability of quantitative determinations of microorganisms in food and feed products is large (Lombard, 2006).

Protocols for sampling of silage often suggest sampling during harvest or sampling from a freshly exposed cutting front. The former makes representative sampling from the entire stack theoretically

possible, e.g. by regular sampling during unloading of the crop, but cannot take into account the exact effects of the fermentation process in the specific stack. The latter gives a good estimate of the silage presently being fed out, but does not represent the entire stack. Analysis of a couple of full-depth drill core samples taken different places in the stack is an intermediate solution giving post-fermentative samples from a larger proportion of the silage stack. Unfortunately, sampling of silage stacks is also restricted as perforation of the silage cover leads to intrusion of oxygen which induces aerobic degradation of the silage (McDonald et al., 1991). The number of invasive samples should therefore be kept to a minimum. Optimal silage sampling is a careful balance between obtaining a representative sample and minimizing the impact of the sampling procedure. This dilemma is often considered but has seldom been systematically examined and documented.

To our knowledge very little information is published on the variation of microbial and feed value parameters within silage. A study of 10 big bales of grass silage revealed large variations in mould and yeast counts both within and between bales (O'Brien et al., 2006). Considerable variation in the fermentation products and bacterial DNA profiles have been documented for big bales of grass silage (Naoki and Yuji, 2008). Another study of the variations in nutritive value within indoor farm silos of grass and whole-crop barley silage (Pedersen et al., 2000) concluded that between one and 17 samples would have to taken from the silage silos, to obtain the desired certainty of the analysis results. A study of the chemical composition and moisture content from 10 samples taken from the cutting front of a wilted pasture silage showed variation for volatile fatty acids, soluble sugars, lactic acid and dry matter content (Haslemore and Holland, 1981), while an early description of silage sampling by coring (Alexander, 1960) describes the error between opposing ends of silage stacks as the largest component of the total sampling error.

To supplement a larger study of fungal spoilage of maize silage (Storm et al., 2009), repeated sampling was conducted on 5 maize silage stacks to study the intra stack variation. Individual samples and sub-samples were analysed for nutritive value and contents of microorganisms. The purpose of the study was to determine whether variations within silage stacks have a significant

impact on the overall measurement uncertainty of individual silage sample. The microbial analysis variance originating from individual steps of the microbial analysis procedure was also evaluated. On the basis of these estimates of variance the number of samples from one stack, which is required to obtain predetermined confidence intervals for stack means, was calculated.

Materials and methods:

Field sampling

Five dairy farms with bunker silos of maize silage, all in the vicinity of Viborg, Denmark, were selected for the measurements. One stack was prepared with a homofermentative bacterial additive, the others without any additives. Overall stack dimensions were 20 – 25 meters in length, 8-15 meters in width and 2.5 – 4 meters in height. All stacks were covered with plastic and protective nets weighed down by tires.

Protective nets and tires were removed from parts of the silage stack to uncover 15 meters in length of the plastic covered surface of the silage stacks. On each stack a square area of approximately 8 m x 12 m was marked and 5 sampling spots chosen by random selection of X and Y coordinates in the marked square. At each spot a primary sample was taken with an automated steel borer (Ø=50 mm; Frøsalget, Brørup, Denmark) in full depth of the stack, except the 0.3 m nearest the concrete bottom. 3 stacks had a layer of grass silage below the maize silage. In these cases samples were taken in full depth of the maize silage. Each primary sample (2-3 kg) was collected in a large plastic bucket and mixed thoroughly by hand before taking out two secondary samples of 500-800 g intended for microbial and nutritional analysis. Each secondary sample was put in a 4 L plastic zipper bag and squeezed to exclude as much air as possible before sealing.

Microbial analysis

All samples for microbial analysis were stored between 0 and 5^{o}C until sample reduction and analysis was performed. Additional reductions in sample size for the microbial analysis was done aseptically

using gloves and utensils cleaned with 70% ethanol. Samples were mixed well by hand, spread in a cone-shaped pile and divided into four quarters. Two opposing quarters were removed and the process repeated if necessary. One of these tertiary samples was mixed well by hand and distributed in a 2-3 cm thick layer on a sterile plastic bag covering a steel tray marked with a 5x5 cm grid. The distribution was performed one thin layer at a time. Final selection of the 40 g quaternary samples required for microbial analysis was obtained by transferring the silage from one randomly chosen square of the marked grid at a time to a sterile plastic-bag, until 40 g was reached. 360 g of sterile 0.1% peptone water was added and the sample homogenized for 2 min, medium speed in a Stomacher laboratory blender (Seward Medical, London, UK). Serial dilutions inoculated by spread plate technique was performed with 0.1% peptone water according to Samson et al. (Samson et al., 2002).

Repeats were performed on different levels of the sample reduction and analysis procedure to estimate the variance of the microbial parameters between randomly chosen samples at each level of the procedure. The total number of samples was: 5 farms (level farm), 25 primary samples (level 1), 30 secondary samples (level 2), 35 quaternary samples (level 3 and 4 combined), 40 dilution series (level 5), and 80 plate series (level 6). Each plate series was performed on 4 different growth media, one for lactic acid bacteria (LAB), one for yeasts and two for filamentous fungi.

LAB were enumerated on Mann, Rogosa, Sharp (MRS) medium (Oxoid, Greve, Denmark) containing 50 $mg \cdot L^{-1}$ nystatin. Nystatin was added as a concentrated aseptic solution of the compound in 70% ethanol just prior to pouring of the plates. Plates were incubated for three days at 25°C under anaerobic conditions. Dilutions corresponding to 10^{-4} – 10^{-9} g silage per Petri dish were plated.

Yeasts were enumerated on malt yeast glucose peptone plates (MYGP) with antibiotics after 4 days incubation at 25°C. The medium contained 10 $g \cdot L^{-1}$ D(+)-glucose, 5 $g \cdot L^{-1}$ bacto peptone, 3 $g \cdot L^{-1}$ yeast extract, 3 $g \cdot L^{-1}$ Bacto malt extract, 20 $g \cdot L^{-1}$ agar, 50 $mg \cdot L^{-1}$ chloramphenicol and 50 $mg \cdot L^{-1}$ chlorotetracylin. Dilutions corresponding to 10^{-2} – 10^{-8} g silage per Petri dish were plated.

Filamentous fungi were enumerated on V8-juice agar plates (V8) and dichloran glycerol 18% agar

159 plates (DG18) (Samson et al., 2002) both containing chloramphenicol (50 mg·L^{-1}) and
160 chlorotetracyclin (50 mg·L^{-1}). Dillutions corresponding to 10^{-2} – 10^{-7} g silage per Petri dish were
161 plated and enumeration performed after 7 days incubation at 25°C.

162 Feed value analysis

163 The primary samples for determination of feed value were frozen and subjected to the standard
164 procedure for determination of feed value at Eurofins Steins laboratories, Holstebro, Denmark. Dry
165 matter (DM) content was analysed by drying at 60°C (NorFor, 2007) without correction for loss of
166 volatiles. Ash was analysed according to 71/250/EEC (EC Commission, 1971). Crude protein (g·kg DM^{-1})
167 crude fiber (g·kg DM^{-1}), starch (g·kg DM^{-1}), NDF (g·kg DM^{-1}), Organic matter digestibility (OMD),
168 pH, lactic acid (g·kg DM^{-1}), acetic acid (g·kg DM^{-1}) and ammonia-N (g·kg total N^{-1}) were determined
169 by NIR.

170 Statistical analysis

171 All microbial counts of CFU·g $silage^{-1}$ were logarithmically transformed (base-10) to logCFU prior to
172 statistical analysis. Data was analysed with SAS 9.1 (SAS, 2003) using the PROC MIXED procedure on
173 a hierarchical model with six random effects: farm (F_i), primary sample ($L1_i$), secondary sample ($L2_i$),
174 tertiary + quaternary sample ($L3\text{-}4_i$), dilution series ($L5_i$) and plate series ($L6_i$). Assuming all random
175 effects to be independent and normally distributed ($P_i \sim N(0, \sigma_i^2)$) the total variance was calculated
176 as:

$$\sigma_{total}^2 = \sigma_F^2 + \sigma_{L1}^2 + \sigma_{L2}^2 + \sigma_{L3-4}^2 + \sigma_{L5}^2 + \sigma_{L6}^2 \quad (1)$$

177 Calculations were performed with the restricted maximum likelihood method (method=REML) and
178 degrees of freedom calculated with the Satterthwaite approximation (ddfm=satterth) (SAS, 2003).
179 The compliance of the residuals with a normal distribution was checked by Shapiro-Wilk,
180 Kolmogorov-Smirnov, Anderson-Darling and Cramér-von Mises goodness-of-fit tests in the PROC
181 UNIVARIAT procedure (SAS, 2003) and by visual inspection of histogrammes and normal probability
182 plots. Variance homogeneity was checked by plotting standardized residuals against predicted

183 values and against all variables in the model. The significance of individual random variables in the
184 model was tested with a restricted/residual likelihood ratio test, comparing the difference in
185 negative restricted/residual log-likelihood values of the reduced and the full model to a χ^2_{df}-
186 distribution with df equal to the number of parameters eliminated in the reduced model.
187
188 95% confidence limits ($CL_{0.95}$) for the means of n primary samples from the same stack were
189 calculated as:

$$CL_{0.95} = \pm t_{0.975,n-1} \times s/\sqrt{n} \quad (2)$$

190 *s* was calculated as the square root of the combined standard deviation for step 1 to 6 according to
191 equation (1) (excluding σ_F^2). The target 95% confidence intervals were set from a subjective
192 evaluation of significance for the feed ration and fermentation quality.
193

194 Results

195 The mean values for the microbial and feed value parameters are listed in table 1,2 and 3 together
196 with the estimated standard deviations. The tables also show a calculation of the number of primary
197 samples needed from each stack to determine average values for each parameter within the
198 specified 95% confidence intervals.

199 The number of colony forming units (logCFU) of filamentous fungi was unevenly distributed between
200 the 5 selected silage stacks (Figure 1). Samples from one stack showed average logCFU counts for
201 filamentous fungi of 3.6 and 4.2 on DG18 and V8, respectively, while the other 4 stacks had many
202 samples below the limit of quantification (LOQ) of 2 logCFU·g silage^{-1}. Averages of the quantifiable
203 samples from these stacks were in the range 2.0 – 2.2 logCFU·g silage^{-1}. The assumption of variance
204 homogeneity across farm could therefore not be confirmed for the counts of filamentous fungi. As a
205 consequence the farms were divide in a high and a low fungal incidence group and only farm 3 with
206 high incidence of filamentous fungi was included in the statistical calculations for filamentous fungi.

The actual number of samples used for calculations is therefore only 14 and 15 for V8 and DG18, respectively. The main filamentous fungi were *Penicillium roqueforti*, *P. paneum*, *Mucor spp.* and *Geotrichum sp*. Yeast counts were almost all above LOQ but were also unevenly distributed as seen by the high variance (Table 1). The lowest yeast counts were 2.0 and the highest 7.8 logCFU·g silage^{-1}. As revealed by the residual plot a single plate series had and erroneously low logCFU of yeasts compared to other plate series and samples from the same stack. This plate series was therefore omitted from further data analysis. The numbers of lactic acid bacteria (LAB) were all in the quantifiable range and much more homogenously distributed with a relative standard deviation (RSD) of 8%. The numbers of primary samples necessary to determine mean logCFUs for filamentous fungi on V8, filamentous fungi on DG18, yeasts and LAB with 95% confidence limits of ±0.5 logCFU were calculated as 36, 33, 17, and 5, respectively. With a 95% confidence limit of ±1.0 logCFU the sample numbers were 11, 11, 7 and 3, respectively.

Statistical tests of the individual variance components of the microbial method of sub-division and analysis revealed that variance contributions from the secondary sampling and the dilution series were insignificant for yeast and LAB ($P>0.5$) (Table 1). After appropriate stepwise reduction of the model to include only farm, primary sampling, and the tertiary/quaternary sampling steps of the laboratory subdivision all these model components were highly significant for yeasts and LAB ($P<0.0001$). For the filamentous fungi from farm no. 3 the same pattern was detected on both V8 and DG18: The variation resulting from the secondary sampling and variation between dilution series was insignificant and can therefore be omitted (Table 2). In the reduced models the variances between primary samples and between tertiary/quaternary samples were significant with P-values of 0.048 and 0.015, respectively, for V8, and 0.004 and 0.008, respectively, for DG18.

The feed value parameters did not vary within stacks to the same extent as the microbial parameters. The relative standard deviations for primary samples (RSD_{L1}) were in the range 1 to 11%. The parameters with the smallest RSD_{L1} (DM, crude protein, OMD, and pH) would require 3 to

5 samples from an app. 96 m2 area to determine the average values within the set 95% confidence limits. For parameters showing larger variation between primary samples (starch and acetic acid) 7 and 5 samples would be required, respectively, within the limited area examined in this study. For crude fiber, starch, NDF and OMD, the intra stack variations were larger than the variation between farms.

Discussion

Microbial parameters

Significant variation between samples from the same stack was detected for all the microbial parameters. The occurrence of filamentous fungi determined as colonies on V8 and DG18 and yeasts on MYGP media showed relative standard deviations for primary samples from the same stack of 36, 39 and 20 %, respectively. This reveals that it is very difficult to formulate general conclusions on the occurrence of fungi in a whole silage stack from a few sample cores. With triplicate sampling from stack no. 3 in the study, the 95% confidence intervals of the means of mould counts on V8 and DG18 were ±3.8 and ±3.5 logCFU, respectively.

Design of the study

The study was limited to 5 stacks and 5 primary samples per stack, as this was the maximum number of samples which could be processed. Five stacks are too few stacks to represent an average Danish maize silage stack. Therefore, to decrease between-stack variation, 5 similar silage stacks were selected for the study, which limits the applicability of the results to this type of stacks. However, the results may very well be applicable for comparison of similar stacks of a different type. Yeasts and fungi are both believed to play an important role during aerobic deterioration of silage (McDonald et al., 1991) and maize silages have been shown almost always to contain filamentous fungi (Pereyra et al., 2008; Storm et al., 2009). The very low amounts of fungi in 4 of the sampled stacks further limit the interpretation of the fungal results. These results can therefore not be extrapolated to other stacks, but illustrate heterogeneity in occurrence of fungi within a stack. With

a smaller initial dilution factor of the samples during enumeration, count of fungi could most likely have been obtained from more of the collected samples. Yeasts could be enumerated in almost all the collected samples and are hence estimated with higher certainty. Both groups showed a highly variable distribution within the reported silage stacks.

To our knowledge there are only a few publications reporting on the variation of microorganisms within silage stacks, but there are surveys stating average contents and standard deviations i.e. estimating the total variation between random samples from different stacks, and there are experiments where repeated sampling of several silage units is reported with the estimated standard deviations. The detected averages of fungi are comparable to other enumerations of silage mycobiota (Auerbach et al., 1998; Amigot et al., 2006; Storm et al., 2009). Others report a skewed distribution between farms with many yeast counts below the limit of detection (O'Brien et al., 2006; Rossi and Dellaglio, 2007). In both these cases samples are treated within a few hours, minimizing the impact of the unavoidable aeration on the mycobiota. In the present experiment the microbial analysis was performed within a few days from collection of the samples. Seale et al. (1990) suggest a maximum storage time of 24 hours, which was not possible in this study. Some changes in the microbiology of the silage compared to freshly taken samples must therefore be assumed. All samples were however treated similar and all samples from each farm were analysed on the same day.

Filamentous fungi and yeasts

The calculated variances correspond well with the standard deviations reported for total fungi in maize silage by Pereyra et al. (2008), yeast and mould CFUs within big bales of grass silage (O'Brien et al., 2006) and total variance for yeasts in multiple samples from 6 bales of grass silage by Naoki and Yuji (2008). But comparing big bales of grass silage with trench silos of maize silage should be done with caution. In a previous examination of 98 maize silage samples from 20 farms at five different times of the year (Storm et al., 2009) the total variance corrected for effect of time was 1.6, 1.6 and 1.8 logCFU for V8, DG18 and MYGP, respectively. These variances for filamentous fungi were

close to the presently detected but for yeast the value is lower. This could be because the samples from previous experiments were all taken approximately 1 m from the cutting front. According to Pitt and Muck (Pitt and Muck, 1993) these samples may thus have been exposed to higher concentrations of oxygen which may explain the slightly higher mean value of yeast and the lower variance.

Lactic acid bacteria

LAB were much more homogenously dispersed than the fungi with an overall RSD of 8%. The detected numbers of LAB and fungi are in range with other examinations of silage microbiology (Dellaglio and Torriani, 1986; McDonald et al., 1991; Rossi and Dellaglio, 2007; Storm et al., 2009). The lower counts of LAB registrered by Lin et al. (1992) can be explained by their use of Rogosa SL medium for enumeration, which gives lower counts than MRS (Seale et al., 1990). Comparably low logCFUs of LAB in big bales of grass silage were also reported by Naoki and Yuji (2008). The pH of these silages was rather high (approximately 6) and the microbial environment of grass in big bales is very different from maize in trench silos, so direct comparison is not possible. The total variance on LAB CFUs was lower in this case than experienced by others (Dellaglio and Torriani, 1986; Rossi and Dellaglio, 2007; Naoki and Yuji, 2008; Storm et al., 2009). The small number of very similar silage stacks in the present experiment was most likely the cause of the relatively low stack-to-stack variance.

In all comparison of microbial results it must be taken into consideration that growth media and conditions have a large influence on the results. LAB were cultivated on MRS as a good general medium for this group of bacteria (Seale et al., 1990). Anaerobic conditions were chosen to simulate the atmosphere of a silage stack and suppress growth of filamentous fungi. As the silage samples were exposed to atmospheric air during sampling and handling in the laboratory, most of the obligate anaerobic LAB may have diminished. The CFUs on MRS are therefore most likely to represent the group of facultative anaerobic LAB. As they were cultivated at 25^{o}C both mesophile and thermophile LAB were able to grow (McDonald et al., 1991).

Sample reduction

Non-representative mass reduction can introduce large error components and devices like riffle splitters or revolving splitters are recommended devices (Gerlach et al., 2003; Petersen et al., 2004). The use of a riffle splitter was tested on maize silage samples but the particle size distribution rendered it impossible. The presence of just a few 5-10 cm long particles easily blocks the splitter chutes. An empirical rule of thumb states that chutes must be wider than three times the maximum particle size, i.e. in this case more than 30 cm (Petersen et al., 2004). Such a construction would have been extremely oversized for samples of a few kg and the described mass reduction protocol based on quartering and incremental sampling was applied.

This method of sub-division did have a significant effect on the statistical model for all microbial parameters, proving that two samples extracted in the same way are not similar. The microbial analytical procedure does however appear to work equally well to other methods as the combined standard deviations for step 2-5 and the residual standard deviation (step 6) are all comparable to the standard deviations for sub-sampling and residual registered by the International Organization for Standardization (ISO, 2006) for coarse materials like hazel nuts, corn flakes, dried figs and grated celeriac. The bias of the method is not estimated. This would require either a reference method giving the true result or a certified reference material for microorganisms in silage, neither of which exist (ISO, 2006).

Feed value parameters

The intra-stack variations for feed value parameters were more moderate than the variations in fungal counts but still of a considerable magnitude. OMD is the most essential parameter for optimization and composition of the feed ration. Calculations in DLBR NorFor (DLBR NorFor, 2009) shows that a deviation of 0.02 on OMD from the analysed value will affect the milk yield with approximately 0.7 kg ECM per day for a typical dairy cow. Even though OMD is the parameter with the smallest RSD between primary samples it would require 3 samples from one stack to be 95%

certain that the mean OMD coefficient did not deviate more than ±0.02. This emphasizes the necessity of taking more than just one sample of a silage stack.

Comparing to the present study, Haslemore and Holland (1981) detected larger variations in the chemical composition of a freshly exposed vertical face of a stack of wilted pasture silage. The ten samples were however taken at different vertical positions in the stack which may have a large effect on the chemical composition (Muck and Holmes, 2000). Large variations within silos were also detected by Pedersen et al. (2000) analysing samples of grass and whole-crop barley silage at high and low DM levels. Their relative standard deviations within silos were from 3 to 17%, lowest for OM and highest for starch. The absolute standard deviations of their study were larger than detected in the present study, which can be explained by the different crops and different DM levels. The pattern with a high relative stack-to-stack variation for starch and a low for OMD is however parallel to this study. Alexander (1960) detected comparable standard errors for DM (10.4 $g \cdot kg^{-1}$) and crude protein (5.7 $g \cdot kg^{-1}$DM) in a study of two cores from each of 32 silage pits. These results are not directly comparable to the present experiment as they refer to samples intentionally taken to cover large spatial variation. The estimated variance thus covers both spatial variance, sampling variance and analytical variance.

Variance contributions

An estimate of the total variance between single samples from different silage stack all over Denmark is obtained from the statistics database at Danish Cattle Federation, Skejby, Denmark. Approximately 3800 Danish maize silage samples were analyzed for feed value in 2008 by the same procedure as employed in this examination (Table 4). According to these statistics the total variance was underestimated in the current study. This is most likely because the variation between farms is underestimated. The five stacks of the study were explicitly chosen to represent one type of silage stack, while the total 2008 variance covers all types of stacks, cover types, harvest times, times of sampling etc. However, for DM, ash, crude protein, crude fibre, OMD and pH the mean values of this study are very similar to the national 2008 mean values. NDF is lower and lactic acid, acetic acid and

361 ammonia-N means are higher in the present study, which may be because the five stacks were
362 sampled 4-5 months after ensilage. Most of the maize silage samples submitted for feed value
363 analysis are taken 1-2 months after ensilage as farmers need to know the feed value prior to feed
364 out.
365 Regardless of these differences the results show that there is considerable variation in feed value
366 parameters between samples from the same maize silage stacks and that several samples should be
367 taken to obtain reasonably certain estimates of the mean values of a whole stack.
368 To minimize costs of analysis the primary samples can be combined to a composite sample
369 representing a physical average of the stack, under the prerequisite that thorough mixing and
370 representative sub-division is performed (Patil, 1995). However, this does not eliminate the
371 laborious and time-consuming collection of many samples which may have a large impact on the
372 preservation of the silage.
373 Another disadvantage of one composite sample opposed to individual samples is, that the
374 unavoidable variation in the method of analysis may have a large influence on the result when only
375 one sample is analysed. The reproducibilities of the feed value parameter analysis ,as documented
376 for the NIR method employed in the present study (Table 4), constitute between 25 and 204% of the
377 inter-stack variation, when calculated as variance. Determination of reproducibility includes day-to-
378 day variation and variation between laboratory operators (ISO, 2006) so samples analysed within the
379 same day and by the same operator should have a smaller standard deviation. This may explain that
380 the variance on the method of analysis exceeds the variance on primary samples from the same
381 stack. None the less the standard deviations of the analytical methods and the root mean square
382 error of prediction for the NIR method (Table 4) are so large that it does not make sense to analyse
383 only one sample from a stack. Separate analysis of several samples is needed to account for the
384 variation originating from both sampling and analysis. Alternatively, more precise methods of
385 analysis should be used.
386

Conclusion

Large variations within maize silage stacks have been demonstrated for microbial parameters. It is therefore clear that values based on one or a few full depth samples from a whole silage stack must be interpreted with great care, as the error margins of such values are large. In particular the distribution of fungi in maize silage is very heterogeneous and more than 11 samples would be required from a silo of maize silage to determine average number of fungal CFUs within a 95% confidence limit of ±1 logCFU. For the feed value parameters 3 to 7 primary samples were needed to reach the target confidence intervals. This is an unfeasibly high number of samples. An alternative or supplementary solution may be regular analysis of samples from the freshly exposed cutting front of silage stacks.

Acknowledgements

The authors wish to thank Niels Toftegaard (Danish Agricultural Advisory Service, Viborg, Denmark) for establishing contact to the farmers, and the farmers for their participation, help and interest. We also thank Niels Bastian Kristensen (Faculty of Agricultural Sciences, Aarhus University) for lending out equipment and advice during planning. The assistance of Jens L. Sørensen during sampling and of Jesper Mogensen in the laboratory is highly appreciated. The studies were funded by the Danish Cattle Federation and the research school FOOD.

406 **Figure 1:** Mean values of the microbial counts of filamentous fungi on V8 and DG18, of yeasts and
407 lactic acid bacteria (LAB) obtained from five separate samples in each of five maize silage stacks.
408 Only counts above the limit of quantification were included. Error bars indicate 95% confidence
409 intervals. Columns without error bars mean that only one sample was above the limit of
410 quantification.
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Tabel 1: Summary data and standard deviations for the log-10 transformed counts of colony forming units of yeasts on MYGP and lactic acid bacteria on MRS detected in maize silage samples. Five samples were taken from each of 5 stacks and repeats conducted at each subsequent level of the sample reduction procedure (L2-L6). Standard deviations for levels with significant effect on the total variation are included.

	n[a]	mean	S_{stack}	S_{L1}	S_{L2}	S_{L3-4}	S_{L5}	S_{L6}	S_{total}
Yeast	76	4.8	1.44	0.95	ns[b]	0.17	ns[b]	0.06	1.73
LAB	80	7.3	0.50	0.31	ns[b]	0.08	ns[b]	0.05	0.60

[a]Counts below logCFU=2 were not detectable.
[b]ns=non significant

Tabel 2: Summary data and standard deviations for the log-10 transformed counts of colony forming units of filamentous fungi in stack no. 3 enumerated on the media V8 and DG18. Standard deviations for levels with significant effect on the total variation are included.

	n[a]	mean	S_{L1}	S_{L2}	S_{L3-4}	S_{L5}	S_{L6}	S_{total}
V8	14	4.2	1.5	ns[b]	0.25	ns[b]	0.14	1.5
DG18	15	3.6	1.4	ns[b]	0.16	ns[b]	0.06	1.4

[a]Counts below logCFU=2 were not detectable.
[b]ns=non significant

Tabel 3: Mean and standard deviations for feed value parameters in 5 samples from each of 5 silage stacks. Standard deviations are given for stack-to-stack variation (S_{stack}), intra-stack variation (S_{L1}) and the variation between single samples from different stacks (S_{total}). The number of primary samples required to obtain a 95% confidence interval with the stated width is calculated with S_{L1}.

	Mean	S_{stack}	S_{L1}	S_{total}	95% conf. interval	No. of samples
Dry matter ($g \cdot kg^{-1}$)	331	16.2	7.9	18.0	10	5
Ash ($g \cdot kg\ DM^{-1}$)	30.6	1.9	1.4	2.4	3	4
Crude protein ($g \cdot kg\ DM^{-1}$)	83.0	4.5	2.4	5.1	5	4
Crude fiber ($g \cdot kg\ DM^{-1}$)	188	6.7	9.7	11.8	10	7
Starch ($g \cdot kg\ DM^{-1}$)	336	18.9	20.8	28.1	20	7
NDF ($g \cdot kg\ DM^{-1}$)	350	0.0	17.1	17.1	20	6
Organic matter digestibility	0.768	0.004	0.008	0.009	0.02	3
pH	3.78	0.13	0.06	0.15	0.2	3
Lactic acid ($g \cdot kg\ DM^{-1}$)	76.88	9.25	3.82	10.01	10	3
Acetic acid ($g \cdot kg\ DM^{-1}$)	20.44	2.66	2.15	3.42	3	5
Ammonia-N ($g \cdot kg\ total\ N^{-1}$)	92	4.7	3.8	6.0	10	3

506 **Table 4:** Mean values and standard deviations (S_{total}) for feed value parameters of the approximately 3800
507 Danish maize silage samples analyzed in 2008 and reported to the Danish Agricultural Advisory Service
508 database. The root mean square error of prediction (RMSEP) for parameters determined by NIR is included as
509 well as the intra laboratory reproducibility standard deviations (S_R) for all feed value parameters.

	Mean [a]	S_{total}[a]	RMSEP[b]	S_R [c]
Dry matter ($g \cdot kg^{-1}$)	335	35.7		6.5
Ash ($g \cdot kg\ DM^{-1}$)	33	8.0		2
Crude protein ($g \cdot kg\ DM^{-1}$)	78	8.3	3.6	2
Crude fiber ($g \cdot kg\ DM^{-1}$)	189	20.2	12.0	9
Starch ($g \cdot kg\ DM^{-1}$)	326	46.5	15.2	14
NDF ($g \cdot kg\ DM^{-1}$)	376	36.2	13.1	11
Organic matter digestibility	0.773	0.018	0.018	0.008
pH	3.78	0.141	0.085	0.03
Lactic acid ($g \cdot kg\ DM^{-1}$)	49	11	4.1	2
Acetic acid ($g \cdot kg\ DM^{-1}$)	14	4.7	5.1	2
Ammonia-N ($g \cdot kg\ total\ N^{-1}$)	39	12	8.0	3

510 [a]Obtained from the Danish Agricultural Advisory Service database (Kjeldsen and Thøgersen, 2009).
511 [b]Adapted from calibration report 2008 for NIR analysis of forages from Eurofins Steins, Holstebro, Denmark
512 (090310/LKS)
513 [c]Standard deviation between individual measurements performed at the same laboratory on different days
514 with different laboratory staff. Data from validation reports from Eurofins Steins, Holstebro, Denmark
515 (Lambert Sørensen, Eurofins Steins, Denmark, personal communication)
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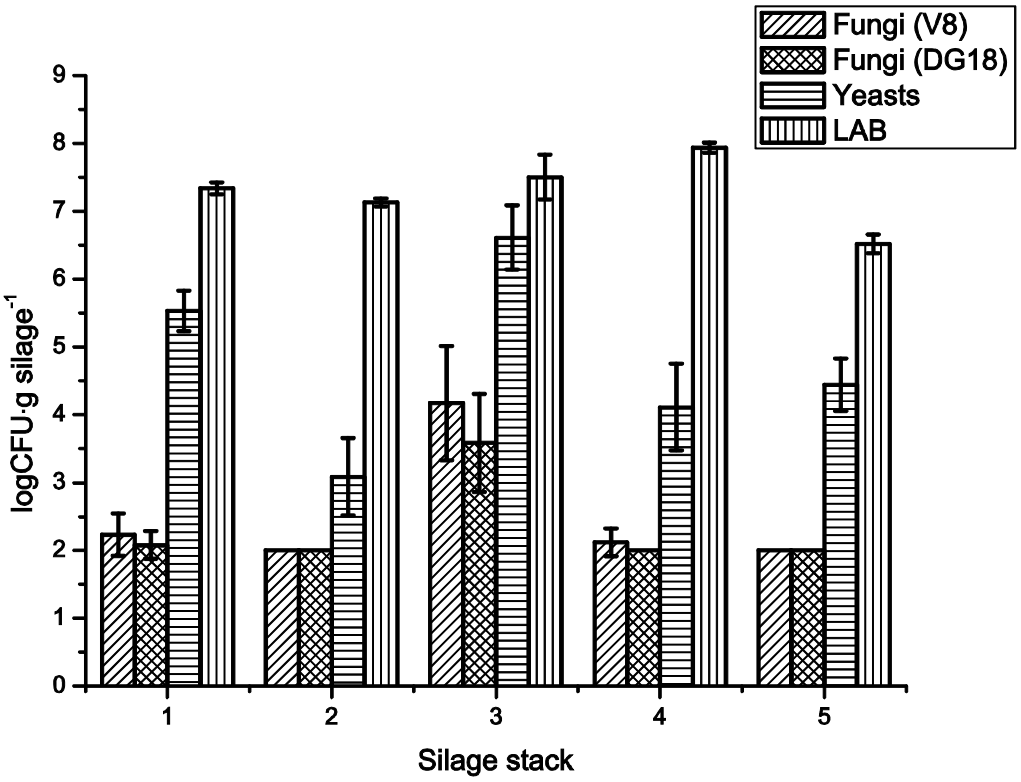

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520 Figure 1

Original paper IV

Green, O., Nadimi, E.S., Blanes-Vidal, V., Jørgensen, R.N., Storm, I.M.L.D., and Sørensen, C.G. (2009). Monitoring and modeling temperature variations inside silage stacks using novel wireless sensor networks. *Accepted by Computers and Electronics in Agriculture.*

Monitoring and modeling temperature variations inside silage stacks using novel wireless sensor networks

Ole Green[a], Esmaeil S. Nadimi[b]*, Victoria Blanes-Vidal[b], Rasmus N. Jørgensen[b], Ida ML Drejer Storm[c] and Claus G. Sørensen[a]

[a]Department of Agricultural Engineering, University of Aarhus, Aarhus, Denmark
[b]Faculty of Engineering, Institute of Chemical Engineering, Biotechnology and Environmental Technology, University of Southern Denmark, Odense, Denmark
[c]Technical University of Denmark, Department of Systems Biology, Kgs. Lyngby, Denmark
*Corresponding author: Email: esi@kbm.sdu.dk

Abstract

By monitoring silage temperature at different locations inside silage stacks, it is possible to detect any significant increases in temperature occurring during silage decomposition. The objectives of this study were: (1) to develop novel noninvasive wireless sensor nodes for measuring the temperature inside silage stacks; (2) to design a suitable sensor protection housing that prevents physical and chemical damage to the sensor; and (3) to mathematically model temperature variations inside a silage stack, using system identification techniques. The designed wireless nodes were used to monitor temperatures in a full-sized silage stack over 53 days. Results showed that the wireless sensor nodes accurately monitored the temperature inside the silage stack at depths of 25 and 50 cm and reliably transmitted the measured data through the network; between 98.9% and 99.4% of the packets disseminated from the sensor nodes were successfully delivered to the gateway. The reliable performance of the network confirmed the correct choice of network characteristics (i.e., frequency range of 433 MHz, a handshaking communication protocol, and 10 mW transmission power). The designed sensor housings were capable of withstanding the high loads that occurred during ensiling, storage, and feed-out. Mathematical models estimating the relations between the silage temperatures (at depths of 25 and 50 cm) and air and soil temperatures were obtained. Black-box modeling using the prediction error method (PEM) was selected as the identification method. Among different black-box models such as ARX, ARMAX, Output Error (OE), and Box-Jenkins (BJ), with different model orders, a third-order Box-Jenkins model structure gave the best performance in terms of prediction accuracy. The success rate of the models proposed for silage temperature prediction ranged between 90.0% and 94.3%. Furthermore, there was no

significant autocorrelation remaining in the residuals. The results of this study indicate that the designed wireless sensor nodes could potentially be used for detecting silage decomposition processes and improving the efficacy of silage conservation systems.
Keywords: Wireless sensor networks (WSN); Silage; System identification; Temperature; Decomposition

1. Introduction

Silage is used worldwide as a major component of the dairy feed base. The term "silage" is generally applied to fermented fodder whose structure is appropriate for feeding ruminant animals (e.g., dairy cows) and which has a relatively low energy content. In order to enrich the silage, further ingredients (e.g., corn) are sometimes added. Over the years, production systems have been intensified and herd sizes have generally increased, leading to a lower proportion of pasture and a higher proportion of silage in the diet of dairy cattle. As a consequence, silage is an increasingly important production factor in dairy farms.

The production of high-quality silage by the farmer leads to a lower demand for external transportation of silage and fodder both locally and globally. However, in order to preserve the nutritional quality of the silage, certain essential conditions need to be met during the storage process. Respiration is the primary cause of silage quality loss, and this depends on the supply of oxygen (O_2), heat and water (McDonald et al., 2002). In practice, silage in dairy farms is usually transported from the field to the storage facility and spread out in thin layers. Each layer is then compacted, for instance with a heavy tractor, before the next layer is added. Immediate action must be taken to prevent oxygen entering the silage stack, and therefore, the silage stack is then sealed using airtight covers. Silage temperature is initially increased due to fermentation. After the initial fermentation period is over, silage temperature should be lower and stable, and any significant increases in silage temperature are associated with aerobic decomposition. The conservation process prevents the digestible matter in the silage from decomposing (McDonald et al., 1991). Failures of the covering system (e.g., tears in the plastic covering, cracks in the walls) cause rapid decomposition of the adjacent silage. In order to ensure adequate preservation of the silage during the entire storage period, it is important to be able to detect potential changes in specific physicochemical properties of the silage that can act as indicators of silage decomposition. During the decomposition process, the dry matter breaks down into H_2O and CO_2 with a release of heat

which is not possible to detect under the sealed covers (McDonald et al., 1991). McDonald et al. (2002) found that losses of dry matter up to 75% were only visible to a very small extent. However, by constantly monitoring the oxygen level (Snell et al., 2001), pH or temperature inside the silage during storage, the status as well as the quality of the silage can be evaluated (Pippard et al., 1996).

Traditional invasive monitoring systems to evaluate the condition of the silage have been used by Pahlow (1984), Williams et al. (1997), McDonald et al. (2002), Osman et al. (2002), and Snell et al. (2001). Measurements of the gas concentration in the silage have been obtained using two different experimental approaches. Pahlow (1984) perfused a mixture of air and CO_2 through silos at a laboratory scale. Using this method, the daily amount of O_2 getting into the silo was determined. Continuous measurement of the O_2 concentration inside the silage stack was carried out by Snell et al. (2001). Williams et al. (1997) equipped bunker silos with gas sampling points. Each of these consisted of a steel container to which a sampling loop (inlet and outlet) was attached which was led out of the bunker and connected to CO_2 and O_2 meters. Both methods (Snell et al., 2001; Williams et al., 1997) were complex and depended on a gas flow, which does not occur to any great extent in compacted silage. The first approach (Snell et al., 2001) resulted in defined conditions, but no measurements of the actual conditions inside the stack were possible. Several recent studies have investigated the electromagnetic properties of the silage and their relationship with moisture content (Barnett and Shinners, 1998; Lawrence et al., 1999; Martel and Savoie, 2000; Osman et al., 2002; Savoie et al., 2000; Shinners et al., 2000; Snell et al., 2000). For instance, parallel plate capacitance sensors were employed by Osman et al. (2002) to measure the moisture content of the silage. Capacitance-type sensors are widely used because of their relatively low cost. The main drawback of these monitoring systems is their negative impact on the preservation of the silage stack. Traditional invasive monitoring systems are usually destructive of the airtight sealing of the silage stack, causing silage to come into contact with O_2, resulting in decomposition of its digestible matter. Consequently, noninvasive novel monitoring systems, such as wireless sensors capable of precisely measuring silage quality parameters, are preferable.

Wireless sensors have been used for different aspects of agricultural measuring, monitoring and control (Wang et al., 2006), such as precision irrigation, environmental field data collection systems, automated fertilizer applicators, and animal behavior monitoring (Damas et al., 2001; Kim et al., 2006; Nadimi et al., 2008; Schumann et al., 2006; Vivoni and Camilli, 2003). Wireless monitoring systems could potentially be applied to measuring quality parameters of the silage stack. Placing networked wireless sensors throughout the storage area would enable the silo to remain

sealed during storage, and could facilitate long-term data collection at scales and resolutions that are better than those obtained using traditional methods. A wireless sensor's intimate connection with its immediate physical environment allows each sensor to provide detailed information that is difficult to obtain through traditional instrumentation such as invasive electrodes or probes. The integration of local processing and data storage allows sensor nodes to perform filtering and data analysis, as well as to apply application-specific aggregation. The ability to communicate not only allows sensor data and control information to be communicated throughout the network of sensor nodes, but also allows nodes to cooperate in the performance of more complex tasks, such as statistical sampling, data aggregation, and system health and status monitoring. Low-power radios with well-designed protocol stacks allow generalized multi-hop communication among network nodes, rather than single-hop communication (Nadimi et al., 2008). The computing and networking capabilities allow sensor nodes to be reprogrammed or re-tasked after deployment in the field. In addition, sensor nodes have the ability to adapt their operation over time in response to changes in the environment. In the design of wireless nodes, communication reliability and low energy consumption are two important factors to be considered. To our knowledge, the use of wireless sensor networks to monitor quality parameters inside full-scale silage stacks has not been reported in the literature.

By monitoring silage temperature at different locations inside the silage stack, any significant increases in temperature occurring during silage decomposition can be quickly detected (Fig. 1). It is essential to detect the decomposition process in its early stages, so as to achieve a more effective conservation process. In order to do this, silage temperature variation over time, prior to the decomposition process, should be modeled. Such a model would be useful not only to detect sealing failures but also to characterize silage performance when exposed to O_2, as the temperature history of the silage during anaerobic storage affects its aerobic stability (Ashbell et al., 2002).

Silage temperature variations after fermentation are a function of air and soil temperatures. A model describing these relationships can be constructed using different modeling techniques. Finite element and finite volume methods have been used to model heat and mass transfer in various applications for the agricultural industry (Marra and Romano, 2002; Norton and Sun, 2006; Norton et al., 2007; Blanes-Vidal et al., 2008). System identification techniques have also been widely used for different applications in agriculture (Juang, 1988; Tiano et al., 2007; Elkaim, 2002; Nadimi et al., 2009).

The objectives of this research were: (1) to develop noninvasive novel wireless sensor nodes capable of precisely measuring the temperature inside silage stacks, with high communication reliability and low energy consumption; (2) to design a suitable protective housing to prevent damage to the sensor from physical (e.g., pressure forces during compaction) and chemical stresses (e.g., acidification) during the ensiling and storage periods; and (3) to mathematically model the variations in temperature inside a silage stack measured by the developed wireless sensor system, based on the variations in air and soil temperature, using system identification techniques.

The organization of the paper is as follows, Section 2 presents materials and methods, including the wireless sensor design, protective housing design, and a description of system identification methods. The results of employing the sensor nodes in the silage stack and applying system identification methods to the measured data, and the models obtained in our study are described in Section 3. Section 4 presents the discussions and the conclusions of this study are presented in chapter 5.

2. Materials and methods

2.1. Wireless node

Various wireless standards for monitoring and automation applications have been established, of which the standards for wireless LAN (local area networking), IEEE 802.11b ("WiFi"), wireless PAN (personal area networking), IEEE 802.15.1 ("Bluetooth"), and IEEE 802.15.4 ("ZigBee") are used most widely. All these standards use the instrumentation, scientific and medical (ISM) radio bands, including the sub-GHz bands of 902–928 MHz (USA), 868–870 MHz (Europe), 433.05–434.79 MHz (USA and Europe), and the GHz bands of 2.400–2.4835 GHz (worldwide).

In general, a lower frequency allows a longer transmission range and a stronger capability to penetrate through different materials. Furthermore, radio waves with higher frequencies are easier to scatter. To obtain a long effective transmission communication range with high penetration capability, 433 MHz was selected as the communication frequency in this application.

The sensor unit (nRF9E5) designed in our study consists of a microcontroller, radio, A/D converter, antenna circuit, power unit (battery), temperature sensor, and relative humidity sensor (Fig. 2). The nRF9E5 is a single-chip system with fully integrated RF transceiver, 8051-compatible microcontroller and a four-input, 10-bit, 80 ksps (kilo samples per seconds) AD converter. The

circuit has embedded voltage regulators, which provides maximum noise immunity and allows operation on a single 1.9–3.6 V supply.

The transceiver of the system automatically handles preamble, address, and CRC (cyclic redundancy check). The RF transceiver is accessed through an internal parallel port or an internal SPI (serial programmable interface). The data-ready, carrier-detect, and address-match signals can be programmed as interrupts to the microcontroller or polled via a GPIO (general purpose input-output) port. The nRF9E5 has a radio transceiver for the 433 MHz ISM bands with Gaussian frequency shift keying (GFSK) modulation at a data rate of 100 kbps. The transceiver consists of a fully integrated frequency synthesizer, a power amplifier, a modulator, and a receiver unit. Output power and frequency channels and other RF parameters are easily programmable by use of the on-chip SPI to the nRF9E5 core. For power saving, the transceiver can be turned on and off under software control. An important aspect of the nRF9E5 node (the operating system of the microcontroller) is its ability to set low-level hardware functionality to achieve low-power sleep states. Sensor nodes are expected to spend most of their time sleeping, and only periodically sample, compute, and communicate, in order to optimize the system lifetime requirements. Minimizing power in sleep mode involves turning off the sensors, the radio, and putting the processor into a deep sleep mode.

In this research, each sensor node acted as a transmit-only device in a single-hop broadcast network and the data was received by a gateway node. To enhance communication reliability, each sensor node actively participated in handshaking communication (Lewis, 2004). Therefore, acknowledgment messages were sent back to the originating node when the sensor messages were received by the gateway. The acknowledgment messages might include information relevant for network re-tasking purposes such as modifications in the network sampling rate. The selected sampling rate for both sensor measurement and packet dissemination was 0.1 Hz, as the temperature in the silage stack varied slowly.

In order to increase packet reception rate and network connectivity beyond the single-hop connectivity options, the sensors may form a multi-hop wireless network by forwarding each other's messages. Using a multi-hop topology vastly extends connectivity options. If appropriate, the network can perform in-network aggregation (e.g., reporting the average temperature across a region). In this research, multi-hop connectivity, as used in modern communication networks, was not utilized, as it was not featured in the operating system of the nodes.

The packet structure routed between the sensor nodes and the gateway is shown in Fig. 3.

"Preamble" is the leader of the packet, "Add" is the address of the receiver, and "Payload" is the valid data of the packet, which includes identification code of the receiver (Tid), identification code of the aimed node (Aid), identification code of the source transmitter (Sid), the marker of the data (Kind), and the data itself (Data). "CRC" is the checking code ensuring the integrity of the message.

2.2. Protective housing

In order to protect the sensor node from damage from physical and potential chemical influences during the ensiling, storage, and feed-out processes, a protective housing was designed (Fig. 4). The plate sticking out of the sphere (Fig. 4, left) holds the battery and sensor in place. The assembled sensor unit (sensor node inside the protective housing) has a diameter of 100 mm, allowing for easy collection of the sensor units, and therefore does not endanger the feeding process of the animals.

Based on an analysis of the handling process, the sensor unit needs to withstand a great deal of pressure during stack provision and afterwards in the feed mixer. Hence, small-scale tests to evaluate the sensor unit's tolerance were carried out in the laboratory prior to deployment.

2.3. Silage temperature modeling

System identification is the process of developing or improving a mathematical representation of a physical system using experimental data (Juang, 1988). If the system to be identified can be explicitly represented as an ordinary differential equation with unknown parameters, linear or nonlinear gray-box models can be estimated. Gray-box modeling is useful when the relationships between variables, constraints on model behavior, or explicit equations of change, are known. Otherwise, black-box modeling is needed (Juang, 1988). A black-box model is a flexible structure that is capable of describing many different systems; however, its parameters might not have any physical interpretation.

The system identification process may be performed in the frequency domain or in the time domain. Among various time domain identification techniques, such as correlation analysis, state space modeling, black-box modeling, and time series analysis, black-box modeling has been widely used due to its robust numerical properties and relatively low computational complexity (Tiano et al., 2007; Juang, 1988; Elkaim, 2002).

Depending on various applications, different types of techniques to estimate the model of the system can be utilized. Based on projection techniques in Euclidean space, subspace identification

methods (SIMs) have been one of the main topics of research in system identification (Gevers, 2003). Several representative algorithms have been published, including canonical variate analysis (CVA: Larimore, 1983), numerical algorithm of subspace state-space system identification (N4SID: Van Overschee and De Moor, 1994), and multivariate output-error state space (MOESP: Verhaegen, 1994). The asymptotic properties of these subspace algorithms have also been investigated and consistency conditions of the estimates have been identified (Deistler et al., 1995; Peternell et al., 1996; Jansson and Wahlberg, 1998; Bauer et al., 1999; Bauer and Jansson, 2000; Knudsen, 2001). The advantages of subspace identification methods compared with prediction error methods (PEM) include simplicity of parameterization, better numerical reliability, and modest computational complexity. However, even though the data satisfy identifiability conditions for prediction error methods, there are some drawbacks, such as generating biased estimates for errors in variables and inapplicability for closed-loop data (Ljung and McKelvey, 1996; Forssell and Ljung, 1999), which should be taken into account.

In our study, a black-box model was selected, as the detailed relationships (differential equations, variables, or constraints) between the temperature of the silage in different layers and the temperature of the surrounding environment and the soil were unknown. A prediction error method was applied in order to obtain unbiased estimates of the parameters of the model.

Black-box models are generally classified into two groups: parametric models and nonparametric models (i.e., correlation analysis and spectral analysis). The step response, impulse response, or the frequency response of the system can be estimated using nonparametric models. In parametric models, however, the parameters are selected as the values that correspond to the best agreement between simulated and measured output. Several model structures, such as ARX, ARMAX, output-error (OE), and Box-Jenkins (BJ), belong to the parametric model category. A significant advantage of parametric models compared with nonparametric models is the imposition of a structure on the system, which leads to compact mathematical formulae with adjustable parameters. These models have attracted significant attention and have been used in a variety of applications (Ljung and McKelvey, 1996).

The general mathematical equation of such a discrete-time linear parametric model is shown in Eq. (1):

$$y(t) = \sum_{i=1}^{N_u} \frac{B_i(q^{-1})}{F_i(q^{-1})} u_i(t - Nk_i) + \frac{C(q^{-1})}{D(q^{-1})} e(t) \qquad (1)$$

where the polynomials B_i, F_i, C, and D contain the time-shift operator q^{-1}. u_i is the ith input, N_u is the total number of inputs, and Nk_i is the ith input delay.

The performance of a model using a prediction error method (PEM) is evaluated by its prediction ability. Therefore, the prediction error of the model presented in Eq. (1) could be expressed as follows:

$$\varepsilon(t, \theta^*) = y(t) - \hat{y}(t|\theta^*) \quad (2)$$

where ε is the prediction error, θ^* represents the parameters of the model to be identified (parameters of the polynomials A, B_i, F_i, C, and D), and y and $\hat{y}$ are the measured and the estimated outputs, respectively.

A model generating the smallest value of prediction error would be considered to be the best representative of the system. A standard performance index, which is convenient both for computation and analysis and minimizes the prediction error, is shown in Eq. (3) (Ljung, 1999):

$$J_N(\theta, Z^N) = \frac{1}{2N}\sum_{t=1}^{N}\varepsilon^2(t, \theta) \quad (3)$$

where J is the performance index, θ represents the true parameters of the system, which are unknown, Z is the data set (inputs and outputs), and N represents the data length. Using Eqs (1), (2), and (3), the parameters of the system are estimated as follows:

$$\theta_N^* = \arg\min J_N(\theta, Z^N) \quad (4)$$

In order to validate the estimated parameters, a subset of the dataset should be used to evaluate the performance of the identified model (validation process). The model estimation process was performed in MATLAB 7.4.0 (R2007a) using the "system identification" toolbox.

2.4. Experimental setup

The experiment was carried out in a full-scale maize silage stack located at Gjorslev Manchen, St. Heddinge, Denmark. The dimensions of the silage stack were 10 m × 50 m × 3 m. During silage

stack preparation, two sensor units (identified as A25 and B25) were placed inside the stack at a depth of 25 cm, and a third sensor unit (A50) was placed at a depth of 50 cm. Measurement locations relatively close to the surface (25 and 50 cm) were selected because spoilage caused by the silage being exposed to air is initiated at near-surface locations. The sensor nodes were programmed to monitor the temperature inside the silage stack and disseminate the packets with a frequency of 0.1 Hz. As the energy of the signal was mainly distributed at two frequency components (very low frequency and at 40 mHz), using Nyquist-Shannon sampling theorem, the sampling frequency should be at least 2 times of 40 mHz which is lower that the selected sampling frequency in this study (0.1 Hz). The maximum transmission power level was 10 mW.

The stack was covered with airtight sealing – two layers of black 0.15 mm Poly Ethylene foil – and the gateway was installed on top of the stack. The maximum distance between sensors and gateway was approximately 1 meter. The experiment was carried out over a period of 53 days, starting in October. In this experiment, values for relative humidity were not monitored.

Climatic data (air temperature and soil temperatures at depths of 10 and 30 cm) were collected on hourly basis from the nearest national climate station (6174, Koge/Herfolge), located approximately 10 km from the experimental farm.

3. Results

3.1. Communication reliability, packet delivery and energy consumption

The performance of a sensor unit (e.g., A25) in terms of communication reliability and packet delivery performance is evaluated, where “1” indicates successful packet delivery to the gateway and “0” indicates packet loss. The packet reception rate during the whole period of the experiment was 98.9%, 99.4%, and 99.3% for A25, B25, and A50, respectively.

Regarding the energy consumption of the wireless nodes, the main sources of energy consumption in the designed nodes were the transmitter and receiver (radio), the processor, and the sensors (temperature and humidity). In nRF9E5 nodes, the radio consumed 11mA in TX mode, 12.5mA in RX mode while the temperature sensor consumed 0.15mA and the humidity sensor consumed 0.77mA. The processor consumed 1.1 nAh for flash read data and 83.3 nAh to write or erase data. Therefore in total, to transmit a packet including the temperature and humidity readings, approximately 12 mA was required. The startup time of the temperature and the humidity sensor was 200 ms and 500 ms respectively. Assuming that the voltage over the poles of the sensors is

within the range of 50% of the nominal and maximum voltage of the battery, it would result in 1.6 V. Consequently, transmission of a packet would consume 0.00373 mAh ($12x1.6 \times 700 \times 0.001/3600$).

The power source in this study was a 3.6 V, 1.2Ah lithium battery. Using a simple calculation, the energy budget available for the entire experiment was 4320 mAh ($3.6 \times 1.2 \times 1000$). Therefore, it can be concluded that transmitting the data packets every 10 seconds (0.1Hz) will result in an operational battery life of 134 days ($4320/(0.00373 \times 360 \times 24)$).

3.2. Protective housing test

In order to evaluate the tolerance of the sensor unit under pressure, a laboratory test experiment was performed on a compression test bench. First, a certain amount of load (1 KN) was imposed on the weak axis of the sensor unit and this was gradually increased until the protective housing was crushed (Fig. 5). The maximum tolerance load for the weak axis was registered as 15 KN. The same test was then conducted on the strong axis of the sensor unit, and the maximum tolerance was found to be 32 KN. After terminating the field test, where the deployed sensor units were exposed to the loads and pressures during stack provision and in the feed mixer, none of the sensor units were broken or deformed, which confirms the suitability of the material and design used for the sensor units.

3.3. Silage temperature monitoring and modeling

Mean, minimum, and maximum temperatures monitored inside the silage stack during the experimental period are shown in Fig. 6. The results showed that temperature variation caused by heat transfer through an intact sealed silage stack (no aerobic processes involved) was slow. For modeling purposes, average daily temperature was considered.

In this study, the model explaining the dynamic behavior of the silage temperature is a multiple-input, single-output (MISO) model. The inputs and the output of the model (i.e., air and soil temperatures, and temperature of the silage at different locations, respectively) are shown in Figs 7 and 8. Application of the present model to countries or seasons in which the solar radiation is intense would require including radiation effects as an input into the model. However, radiant energy from sunlight striking Denmark mainland (latitude 55° 19' 0" N) during October, November and December months is minimal. Meteorological data showed that during most of the day (from 4 p.m. to 9 a.m.), hourly averaged solar radiation is 0 W/m2, and during the central hours of the day,

solar radiation is very limited: 21 W/m2 (9 a.m.), 61 W/m2 (10 a.m.), 87 W/m2 (11 a.m.), 96 W/m2 (12 a.m.), 79 W/m2 (1 p.m.), 44 W/m2 (2 p.m.) and 9 W/m2 (3 p.m.).

Primarily, linear models such as ARX, ARMAX, OE, and BJ with three inputs (air temperature, soil temperatures at 10 and 30 cm depth) and one output were estimated based on the first half of the mean-centered data. Therefore the input-output dataset was detrended prior to the model estimation. The simulated output was then compared to the measured output for the whole data record. Among different models, the Box-Jenkins model structure showed the best prediction performance (Table 1). Table 1 represents the prediction algorithm performance that is based on the use of measured outputs to calculate the future outputs and to estimate the performance of the model. Simulation algorithm that uses the previously estimated outputs to calculate the future outputs were not considered due to stability properties of the algorithm. Tables 2 and 3 show the estimated parameters and the confidence interval of each parameter of the polynomials in Eq. (1) identified by a PEM method. The quantities in parentheses beside each estimated parameter are two times standard errors of each parameter estimate. A 95% confidence limit for each parameter can be calculated as twice the standard error for each parameter estimate. Taking Tables 2 and 3 into account shows that a third- or higher-order model is in perfect agreement with the input-output data. Lower order models e.g. first and second order models were rejected due to the low percentage fit compared to the third and higher order models; while, fourth- or higher-order models were rejected, since pole-zero cancellation would suggest that this could be a consequence of round-off modeling errors.

The prediction performance of each model is shown in Figs 9 and 10, where the silage temperatures measured by A25 and B25 are represented by blue and red curves and the model simulated output by the black curve. Tables 2 and 3 and Figs 9 and 10 show that the models describing the temperature of the silage at depths of 25 and 50 cm predicted the actual measurements with 90.0%, 94.3%, and 92.12% accuracy. Furthermore, using the model residuals, autocorrelation plots, considering as a hypothesis that the autocorrelation is significant, are shown in Fig. 11. There was no significant autocorrelation remaining in the residuals, as the autocorrelation function between the output and the residuals and the cross-correlation function between the input and the residuals were within the 95% confidence intervals.

4. Discussion

In this section, the performance of the designed wireless nodes with regard to three specific aspects (i.e. packet delivery performance, operational life of the sensor nodes and the network density) are discussed.

In the present study, high packet delivery rates (98.9%, 99.4%, and 99.3%) from the sensor units to the gateway were obtained as the result of different decisions taken during the design of the wireless sensor network. Firstly, the selection of the appropriate frequency rate (433 MHz) for the communication among the sensor nodes. Secondly, the participation of the sensor network in handshaking communication. Finally, setting the data transmission with the maximum transmission power level (10 mW).

Regarding the operational life of the sensor nodes (including the protective housings), the experiment carried out in this study showed that the proposed sensor nodes were able to fulfill important requirements related to their future viability under practical conditions. As the objective of this study was to design a novel monitoring system (wireless sensor nodes) nondestructive of the airtight sealing of the silage stack, the wireless nodes should be able to measure and transmit the measurements during the whole period of the experiment without the necessity of removing them from the stack e.g. to change the batteries. In this experiment, the power supply (battery) lasted during the whole experimental period (53 days). Besides, it is also important that the protective housing last without any damage, as any physical or chemical change in the housing might result in invalid sensor readings or communication. The protective housing designed for this application was proven to remain intact after the 53 days experiment.

Finally, evaluation of the viability of the system when used in commercial applications requires information about the density of the network. Based on the environment where the sensor nodes are deployed, the type of interfaces between the wireless nodes (e.g. grass, maize); the communication frequency (e.g. 433 MHz, 868-980 MHz, 2.4 GHz), the routing protocol (i.e. single hop, multi-hop) and the transmission power (e.g. 0mW, 5mW, 10mW), the range of communication and therefore the network density can vary significantly. In this study, the maximum communication range between each sensor node and the gateway (using single-hop routing protocol and the methods and materials reported in this article), was about five meters. Therefore in a silage clamp of the size $10 \times 50 \times 3$ m^3, 20 sensor nodes to monitor a certain depth (e.g. 50 cm) would be adequate. The

density of the network and energy consumption could be reduced if modern communication routing protocols such as multi-hop routing were used.

According to the authors' best knowledge, wireless sensor networks have not been deployed in a real size silage stack and therefore, a direct comparison between the results achieved in this study and results from other studies cannot be carried out. However, the performance of the designed wireless nodes in this specific agricultural application (silage temperature monitoring) was, in terms of packet delivery performance and energy consumption, better that the performance of other wireless nodes designed for other agricultural applications (animal behavior monitoring) (Wang et al., 2006; Nadimi et.al, 2008a, 2008b, 2009).

5. Conclusions

In order to detect silage decomposition at an early stage and to improve the efficacy of silage conservation systems, novel noninvasive wireless nodes capable of measuring the temperature inside silage stacks have been designed and their performance evaluated in a full-scale silage stack.

The designed wireless nodes precisely monitored the temperature inside the silage stack at depths of 25 and 50 cm and reliably transmitted the measured data. The results of these experiments showed that 98.9%, 99.4%, and 99.3% of the packets disseminated from the three tested sensor nodes were successfully delivered to the gateway. In order to protect the sensor nodes against possible physical and chemical damage during ensiling, storage, and feed-out, a protective housing was designed. Results showed that the designed housings were resistant to the high loads occurring during stack provision and feed mixing.

Mathematical models for estimating the relations between the temperature of the silage, at depths of 25 and 50 cm, and air and soil temperatures were evaluated. Among different black-box models, the Box-Jenkins model structure gave the best performance. The proposed models of temperature at different depths were able to predict the temperature measurements with 90.0%, 94.3%, and 92.12% success rates. Furthermore, there was no significant autocorrelation remaining in the residuals, as the autocorrelation function between the output and the residuals and the cross-correlation function between the input and the residuals were within 95% confidence intervals. The estimated model successfully predicted the normal temperature variations of the silage stack using the air and soil temperature as inputs, and so the model could be used to detect the abnormal temperature variations inside the silage stack caused by silage decomposition. The results of this study indicate that the

designed wireless sensor nodes could potentially be used for detecting the occurrence of silage decomposition and for improving the efficacy of silage conservation systems.

Acknowledgments

The authors would like to thank Ole Juul Jørgensen and Johanne Lindstrøm for their great help.

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Figure 1 in PDF

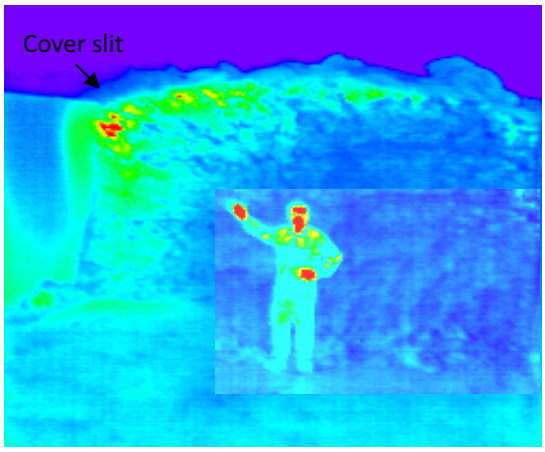

Fig. 1. Temperature contour plot of an unsealed silage stack using a thermal infrared camera, showing high temperatures close to a slit in the cover (Laursen, 2005).

Figure 2 in PDF

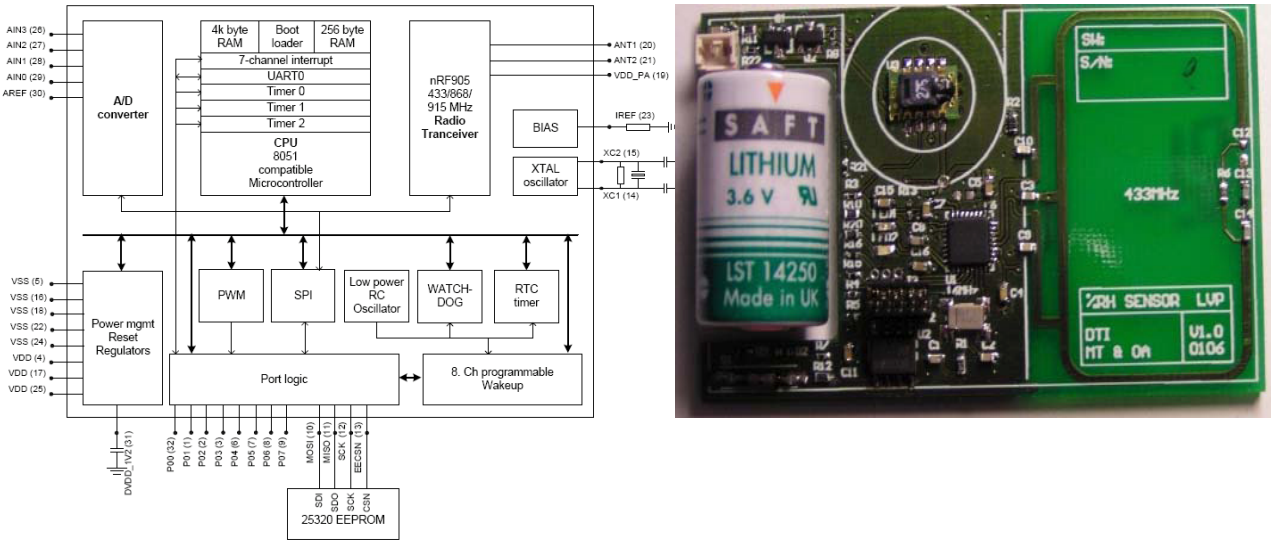

Fig. 2. The designed sensor unit: (a) schematic diagram, (b) actual sensor node.

Figure 3 in PDF

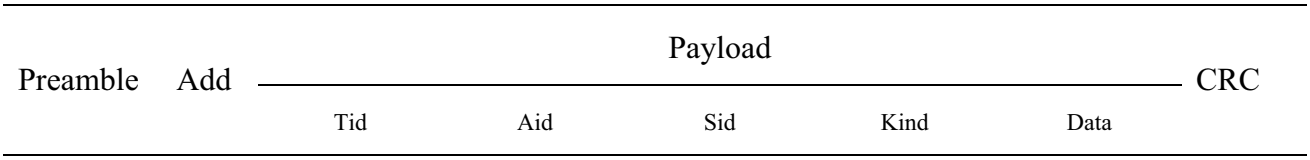

Fig. 3. Data packet architecture.

Figure 4 in PDF

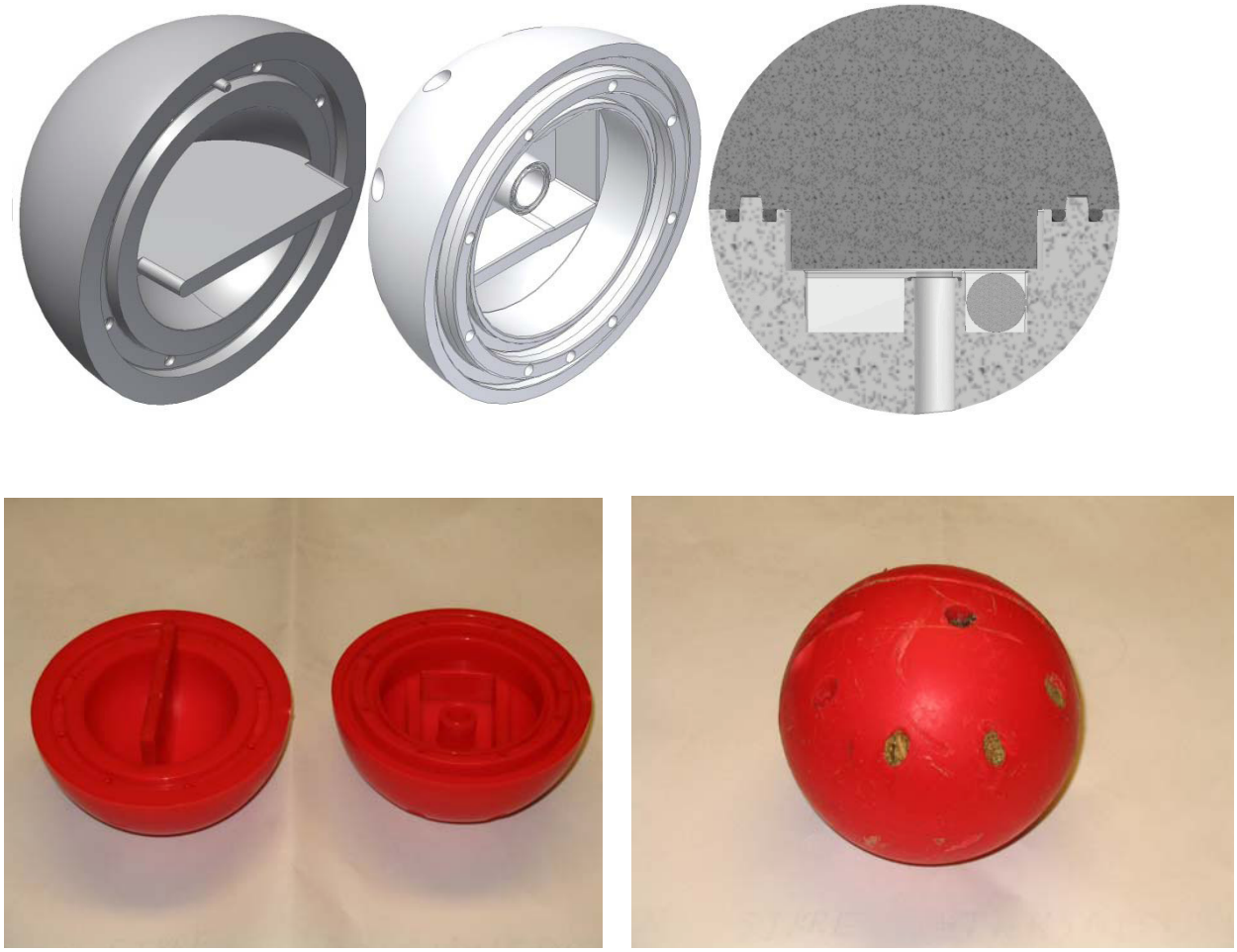

Fig. 4. Schematic diagram and photographs of the sensor's protective housing: top part (left); bottom part (center), and cross-section view (right).

Figure 5 in PDF

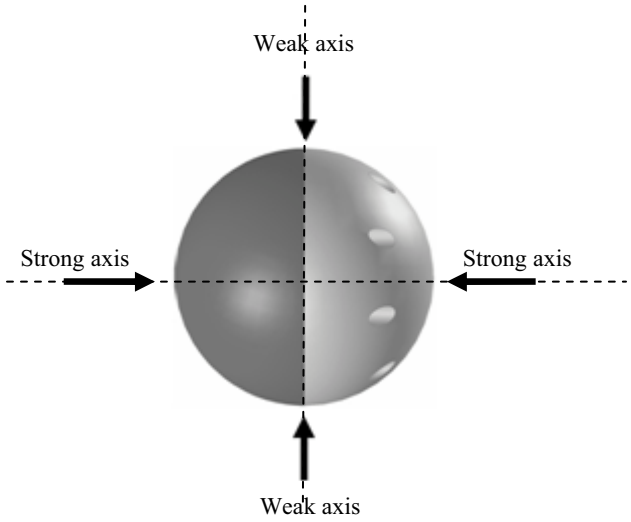

Fig. 5. Direction of the compression forces applied during the experimental laboratory test to evaluate maximum tolerance of the sensor unit.

Figure 6 in PDF

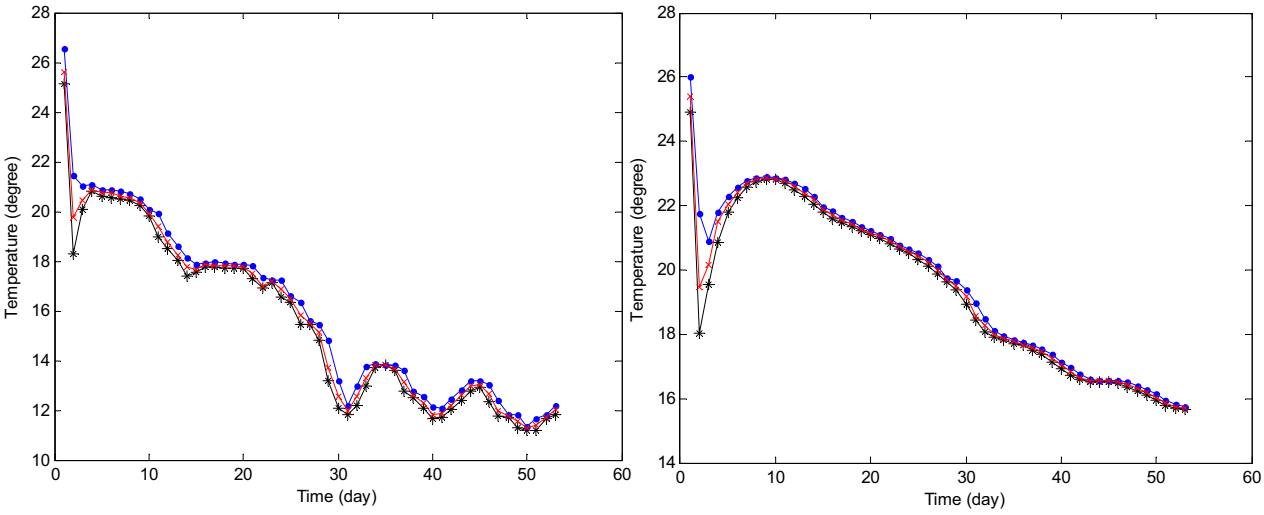

Fig. 6. Temperature variation measured inside the silage stack (B25 on the left and A50 on the right) within the experimental period: minimum (black), maximum (blue) and mean value (red).

Figure 7 in PDF

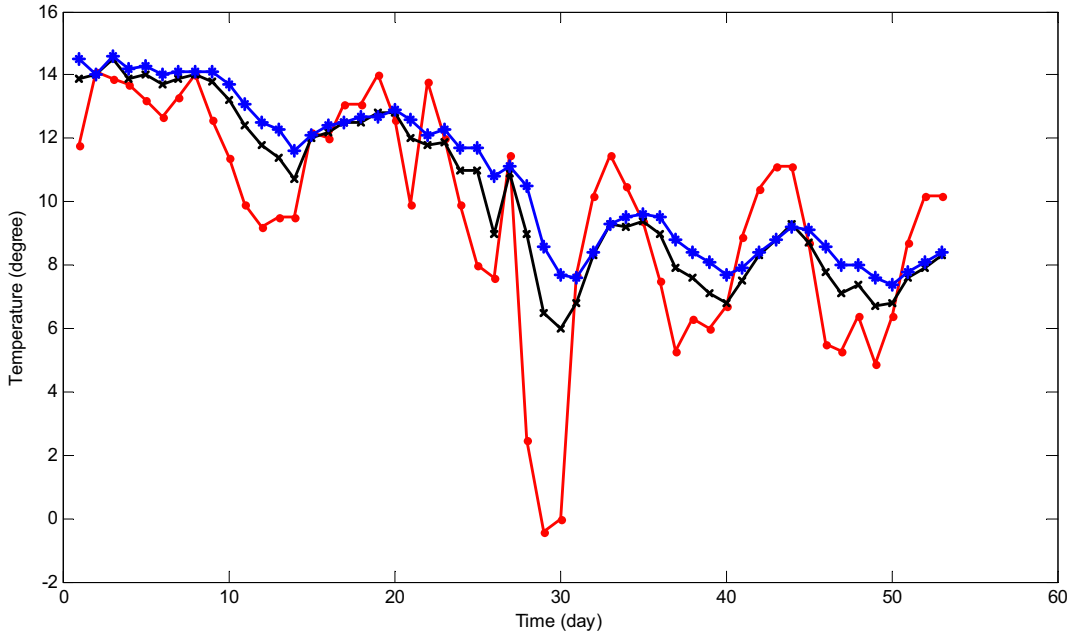

Fig. 7. Measured air and soil temperatures (inputs to the models). The red curve represents daily averaged air temperatures, the black curve represents daily averaged soil temperatures at 10 cm depth, and the blue curve represents daily averaged soil temperatures at 30 cm depth.

Figure 8 in PDF

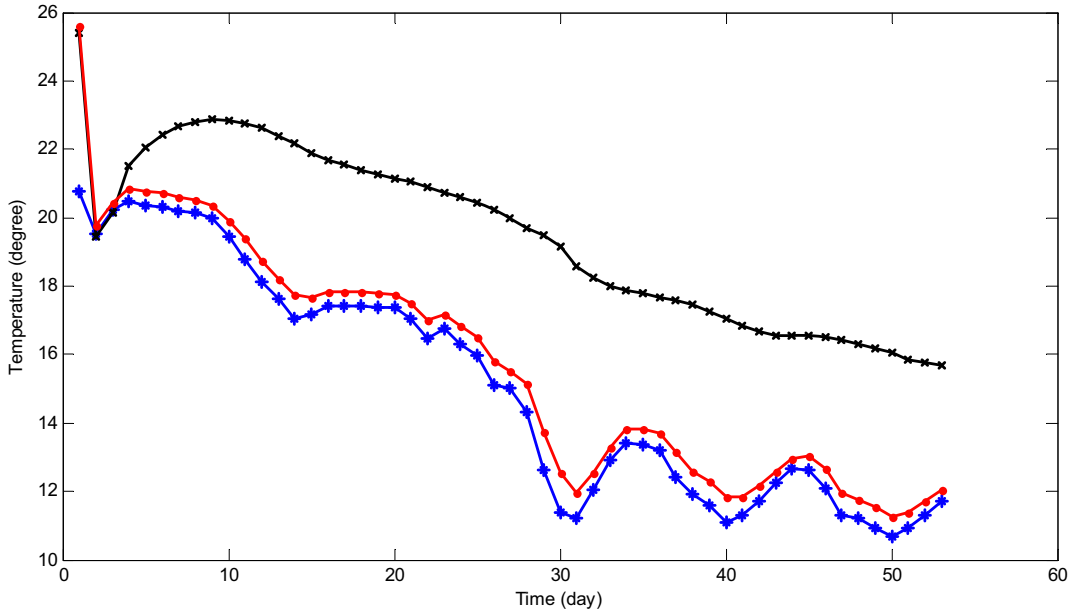

Fig. 8. Measured silage temperatures at 25 and 50 cm from the silage surface. The red and blue curves represent the daily averaged silage temperatures measured at a depth of 25 cm (A25 and B25, respectively). The black curve represents the daily averaged silage temperature measured at a depth of 50 cm (A50).

Figure 9 in PDF

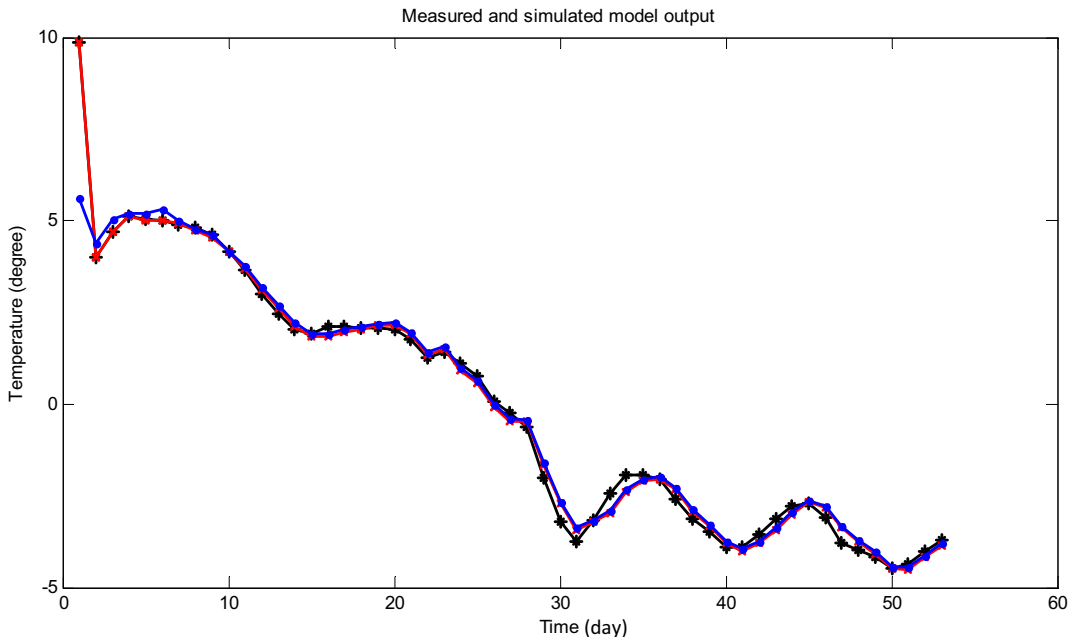

Fig. 9. Silage temperature predicted at a depth of 25 cm (model simulated output (black curve) and actual measurements by A25 and B25 – blue and red curves) without trends of the measurements.

Figure 10 in PDF

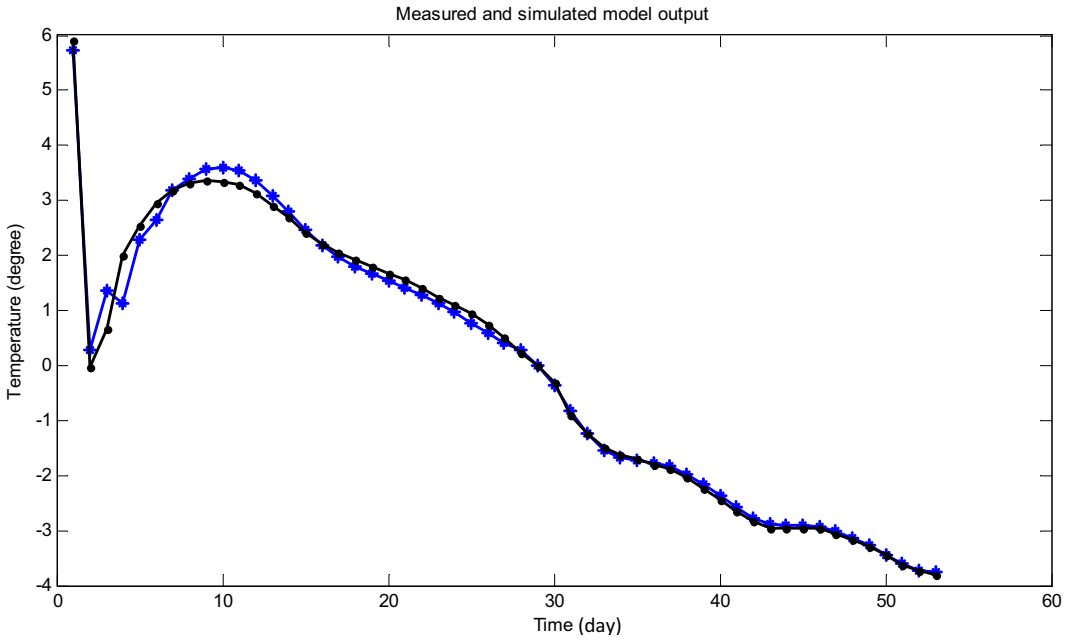

Fig. 10. Silage temperature predicted at a depth of 50 cm (output of the model applied to the data of F6; blue curve) and real system measurements (black curve).

Figure 11 in PDF

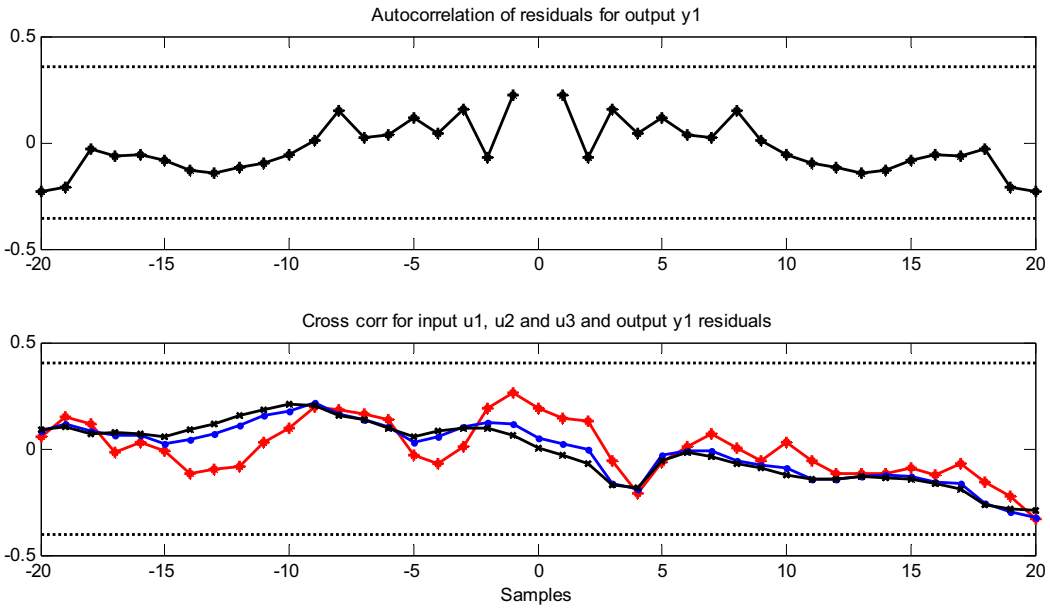

Fig. 11. Autocorrelation function between the output and the residuals (top diagram). Cross-correlation between input 1 (red), input 2 (blue) and input 3 (black) and the residuals (bottom diagram). The dotted lines represent the 95% confidence interval.

Table 1. Performance of different third-order model structures

Third-order model structure fit percentage			
Model structure	**A25**	**B25**	**A50**
ARX	76.30%	80.32%	75.43%
ARMAX	77.85%	82.11%	80.74%
OE	83.27%	91.37%	88.16%
BJ	89.88%	94.31%	92.12%

Table 2 in PDF

Table 2. Estimated parameters for the model explaining the temperature of the silage at a depth of 25 cm (sensors A25 and B25) as a function of air and soil temperatures

	A25/B25		
$B_1(q^{-1})$	$0.128\ (\pm 0.01877)q^{-1}$	Input 1: air temp	$n_{b1}=1$
$F_1(q^{-1})$	$1-0.2591(\pm 0.1294)q^{-1}-0.4392(\pm 0.1333)q^{-2}-0.293(\pm 0.05809)q^{-3}$		$n_{f1}=3$
$B_2(q^{-1})$	$0.1845\ (\pm 0.05622)q^{-1}$	Input 2: soil temp (10 cm)	$n_{b2}=1$
$F_2(q^{-1})$	$1-0.5086\ (\pm 0.3285)q^{-1}-0.2769\ (\pm 0.1934)q^{-2}+0.4048\ (\pm 0.272)q^{-3}$		$n_{f2}=3$
$B_3(q^{-1})$	$-0.2594\ (\pm 0.05728)\,q^{-1}$	Input 3: soil temp (30 cm)	$n_{b3}=1$
$F_3(q^{-1})$	$1+0.436\ (\pm 0.1487)\ q^{-1}-0.9176\ (\pm 0.04781)\ q^{-2}-0.5478\ (\pm 0.1286)\ q^{-3}$		$n_{f3}=3$
$C(q^{-1})$	$1-0.7754(\pm 0.2404)q^{-1}$	Noise model	$n_c=1$
$D(q^{-1})$	$1-1.455\ (\pm 0.1049)\,q^{-1}+0.9263\ (\pm 0.1032)\,q^{-2}$		$n_d=2$
Fit percentage of A25: 89.88% Fit percentage of B25: 94.31%			

Table 3. Estimated parameters for the model explaining the temperature of the silage at a depth of 50 cm (sensor A50) as a function of air and soil temperatures

	A50		
$B_1(q^{-1})$	$0.01449\ (\pm 0.005082)q^{-1}$	Input 1: air temp	$n_{b1}=1$
$F_1(q^{-1})$	$1-0.4301(\pm 0.2304)q^{-1}-0.5559(\pm 0.2258)q^{-2}$		$n_{f1}=2$
$B_2(q^{-1})$	$-0.09408\ (\pm 0.02567)q^{-1}$	Input 2: soil temp (10 cm)	$n_{b2}=1$
$F_2(q^{-1})$	$1-1.598\ (\pm 0.07025)q^{-1}+0.632\ (\pm 0.06694)q^{-2}$		$n_{f2}=2$
$B_3(q^{-1})$	$0.2361\ (\pm 0.04848)\,q^{-1}$	Input 3: soil temp (30 cm)	$n_{b3}=1$
$F_3(q^{-1})$	$1-1.319\ (\pm 0.07279)\ q^{-1}+0.3838\ (\pm 0.0631)\ q^{-2}$		$n_{f3}=2$
$C(q^{-1})$	$1+0.3889(\pm 0.2409)q^{-1}$	Noise model	$n_c=1$
$D(q^{-1})$	$1-0.302\ (\pm 0.08739)\,q^{-1}$		$n_d=1$
Fit percentage of A50: 92.12%			