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Environmental Exposure to Estrogenic Mycotoxins

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Table of contents

Summary

Endocrine disrupting compounds (EDC's) are a serious problem in the environment due to their interferences with animal and human hormone systems, as described for many cases in the past. Most of these cases were caused by industrial chemicals, which were introduced to the environment by human activities. Aside from these synthetic EDC's, also naturally occurring compounds, such as the resorcyclic acid lactones (RALs) are known having endocrine disrupting potential. The most prevalent compound out of the RALs is zearalenone (ZON), which is orders of magnitudes more potent than notorious synthetic EDC's such as DDT, bisphenol A or atrazine. ZON is produced by a variety of *Fusarium* species, in particular *Fusarium graminearum*, which grows on crops such as maize and wheat. To this point, ZON has only been recognized as a problem in the feed context which manifested itself for instance in infertility problems in husbandry animals, in particular in swine. In this thesis the distribution of ZON and its fate with respect to environmental exposure was studied for the first time. As crop fields are the primary source of *Fusarium* toxins we conducted a field study where we cultivated wheat and maize. The crop rotation wheat after maize is known to be favourable for *Fusarium graminearum* infection and therewith ZON contaminated crops were expected. In particular, ZON levels were measured in the wheat- and maize plants, the field soil and the drainage water. In addition, other possible sources such as waste water treatment plant effluents and manure application were investigated. ZON was also quantified in several large rivers throughout the Swiss midlands, with respect to the overall exposure of surface waters.

Very sensitive analytical methods were required for quantification of ZON in various agro-environmental matrices, due to expected concentrations at the trace level. The use of deuterated analogues (D_6-ZON) as internal standard and conventional analytical techniques such as solid phase extraction for concentration, Soxhlet extraction and high performance liquid chromatography coupled with tandem mass spectrometry allowed ZON quantification in the very low ng/L and the low ng/g range in aqueous and solid matrices, respectively. Method detection limits (MDL's) in aqueous samples were 0.5, 0.7 and 0.8 ng/L for drainage, surface and waste water. For solid matrices MDL's were 1.0-8.5, 0.2, 3.7 and 2.0 $ng/g_{dry\ weight\ (dw)}$ for plant material, soil, manure and sewage sludge, respectively. Absolute and relative

recoveries were mostly around 100%, and precision ranged from 3 to 16% for both, aqueous and solid matrices.

ZON concentrations in the wheat- and maize plant organs varied between 100 ng/g and 17 µg/g dry weight. Resulting total ZON amounts in the plants at the time of harvest were 7 and 32 g/ha, depending on the crop and influenced by climatic conditions. After kernels were removed by harvest, ZON amounts associated with plant debris which remained on the field were between 6 and 25 g/ha. Besides ZON contaminated plant debris, manure/dung was another ZON source investigated, because straw material is one of its constituents and ZON can pass the gastrointestinal tract of husbandry animals. Concentrations of ZON in manure and dung ranged from 7 up to 330 µg/kg dry weight. Considering an average manure application practice, between 50 and 150 mg/ha/year would reach agricultural soil. These amounts were in a similar range as the ZON amounts directly washed off from contaminated plants. However ZON amounts present in plant debris on the field after harvest were one to two orders of magnitudes higher than the ones in manure.

Concentrations of ZON in the topsoil layer of our field were between not detectable and 3.8 ng/g dry weight, resulting in total ZON mass of 0 to 5.6 g/ha. These levels were influenced by several overlaid processes such as climatic conditions, crop variety, continuous ZON emission from the plants and their debris, possible degradation processes (not investigated in this study), ZON leaching into the drainage water and sorption processes. Sorption of ZON on the field soil was found to take place mainly on the organic carbon (OC), which is apparent from a fitted water to OC distribution coefficient of 3318 L/kg_{oc}. The predominant sorptivity of OC compared to other soil constituents such as sand, clay minerals or iron oxides was confirmed by the results obtained with ZON sorption experiments to humic acid, goethite and montmorillonite.

The considerable sorption of ZON on soil together with the correlation of the drainage water discharge with the ZON concentrations in the drainage water (“first flush” effect), lead to the conclusion that ZON emission from the field site was mainly driven by preferential flow via macropores. ZON concentrations in the drainage water were in the very low ng/L range with a maximum peak concentration of 35 ng/L. Cumulative ZON amounts emitted from the field via drainage water were between 0.1 and 4.3 mg/ha, depending on the crop cultivated in the respective period. These

amounts correspond to fractions between 0.001 to 0.070% of the initially present ZON amount in the plants.

Our data clearly show that soil is the primary recipient of ZON emitted from *Fusarium graminearum* infested wheat and maize fields. Due to the large dilution of drainage water in surface waters, ZON concentrations will usually drop below detection limit and probably environmentally critical levels, although little is known about ecotoxicological consequences of pulsed exposures. In several hundred investigated surface water samples taken throughout the midlands of Switzerland, ZON was detected in three cases only. Still, in small creeks mainly fed by agricultural runoff, ZON might contribute substantially to the total estrogenicity of such water in case of *Fusarium graminearum* occurrence. In a two month period, besides ZON also another mycotoxin (Deoxynivalenol, DON) was quantified in the drainage water emitted from the field site. Concentrations were between 23 ng/L and 4.9 µg/L. DON levels in Swiss rivers were up to 22 ng/L.

Other mycotoxins might be emitted from *Fusarium*-infested fields as well, as some of them are produced in similar amounts as DON and ZON. In the future, we suggest extending the search for such mycotoxins acting as aquatic micropollutants.

Zusammenfassung

Das Vorkommen von hormonaktiven Chemikalien (endocrine disrupting compounds, EDC's) in der Umwelt stellt für diese ein ernstes Problem dar. In der Vergangenheit hat es viele Ereignisse gegeben, bei welchen industrielle Chemikalien Abnormalitäten bei Tieren verursacht haben. Neben synthetisch hergestellten EDC's gibt es aber auch Pflanzen und Schimmelpilze, die Gifte mit hormonaktiver Wirkung produzieren. Ein dafür bekannter Schimmelpilz ist *Fusarium graminearum*. Dieser wächst auf verschiedenen Getreidearten, wie beispielsweise Weizen, und Mais. Als Stoffwechselnebenprodukt produziert *Fusarium graminearum* resorzyklische saure Laktone (resorcyclic acid lactones, RALs). Der am meisten vorkommende Vertreter der RALs ist das Zearalenone (ZON). Seine Östrogenizität liegt mehrere Grössenordnungen über denjenigen von bekannten synthetischen EDC's wie DDT, Bisphenol A oder Atrazin. In der Vergangenheit hat man die Problematik des hormonaktiven ZON hauptsächlich in der Futtermittelindustrie wahrgenommen und untersucht. Diese zeigt sich zum Beispiel in der Schweinehaltung, wo Fertilitätsprobleme durch ZON kontaminiertes Futter oder Stroh hervorgerufen werden. In dieser Arbeit haben wir das erste Mal die Verteilung und das Verhalten von ZON in der Umwelt untersucht. *Fusarium* infizierte Mais- und Weizenfelder sind primäre Quellen, aus denen ZON in die Umwelt gelangen kann. Deshalb haben wir eine Feldstudie mit der Fruchtfolge Weizen nach Mais durchgeführt. Diese Fruchtfolge ist bekannt für ihre Anfälligkeit für *Fusarium* Infektion, weil *Fusarium* infiziertes Pflanzenmaterial, welches nach der Maisernte auf dem Feld zurückbleibt, den nachfolgenden Weizen infizieren kann. Um den Eintrag von ZON über *Fusarium* infizierte Mais- und Weizenfelder zu untersuchen, haben wir Pflanzen, Boden und Drainagewasser auf deren ZON Gehalte analysiert. Zusätzlich haben wir Gülle- und Klärwasserproben als mögliche Quellen auf ZON untersucht. Des Weiteren haben wir in mehreren grossen Flüssen im Schweizer Mittelland die ZON Gehalte gemessen, um ein Bild der schweizweiten Belastung zu erhalten.

Um ZON in den erwähnten Proben im Spurenbereich nachweisen zu können, waren wir auf sehr empfindliche Messmethoden angewiesen. Deuteriertes ZON (D_6-ZON) und der Einsatz von modernen chemisch analytischen Methoden und Geräten haben dies ermöglicht. Die flüssigen Proben wurden mittels Festphasen Extraktion angereichert und die festen Proben mit Soxhlet extrahiert. Die Extrakte wurden dann mittels Hochleistungs-Flüssigchromatographie (HPLC) getrennt und mit Tandem-

Massenspektrometrie detektiert. Die Nachweisgrenzen für ZON waren 0.5 ng/L in Drainagewasser, 0.7 ng/L in Flusswasser und 0.8 ng/L in Klärwasser. In den festen Matrices lagen diese bei 1-8.5 ng/$g_{Trockensubstanz\ (TS)}$ in Pflanzenmaterial, 0.2 ng/g_{TS} in Boden, 3.7 ng/g_{TS} in Gülle und 2.0 ng/g_{TS} in Klärschlamm. Absolute und relative Wiederfindungen lagen für die meisten flüssigen und festen Matrices im Bereich von 100% und die Präzision zwischen 3 bis 16%. ZON Konzentrationen variierten je nach Getreideorgan zwischen 100 ng/g und 17 µg/g Trockengewicht. Die daraus resultierenden totalen ZON Mengen lagen zwischen 7 und 32 g/ha und waren abhängig von der Getreideart und den klimatischen Bedingungen. Durch die Ernte wurden die Körner jeweils vom Feld entfernt. Die zurückgebliebenen Pflanzenreste auf dem Feld beinhalteten totale ZON Mengen zwischen 6 und 25 g/ha. Weil Stroh ein Bestandteil von Gülle und Mist ist, können diese als zusätzliche ZON Quellen für die Umwelt nicht ausgeschlossen werden. Die Konzentrationen lagen zwischen 7 und 330 ng/g_{TS}. Aufgrund einer durchschnittlichen Düngepraxis gelangen dadurch in einem Jahr zwischen 50 und 150 mg/ha auf landwirtschaftliche. Diese ZON Menge ist vergleichbar mit der Menge, die von *Fusarium* befallenen Mais- und Weizenpflanzen durch den Regen abgewaschen wurde. Die nach der Ernte in Pflanzenresten auf dem Feld verbleibende ZON-Menge war um eine bis zwei Grössenordnungen höher.

Im Oberboden des Feldes lagen die ZON Konzentrationen zwischen der Nachweisgrenze und 3.8 ng/g. Die daraus resultierenden totalen ZON Mengen betrugen bis zu und 5.6 g/ha. Diese Werte wurden durch die klimatischen Verhältnisse, das kontinuierliche Abwaschen von den Pflanzen und Pflanzenresten, den möglichen Abbau (in dieser Arbeit nicht untersucht) und Sorptionsprozesse beeinflusst. Der organische Kohlenstoff (OC) im Boden war der wichtigste Bodenbestandteil in Bezug auf die Sorption von ZON. Dies zeigte der hohe OC zu Wasser Verteilungs-Koeffizient von 3318 L/kg_{oc}. Dieser Befund wurde durch die Resultate aus weiteren Sorptionsexperimenten mit ZON an Repräsentanten von Bodenbestandteilen wie Huminsäure, Goethit und Montmorillonit gestützt.

Die Tatsache, dass ZON relativ stark am Boden sorbierte und dass die ZON Konzentration im Drainagewasser mit dessen Abfluss korrelierte („first flush“ effect), deutet darauf hin, dass ZON vom Feld hauptsächlich durch „preferential flow“ via Makroporen ausgetragen wird. Die ZON Konzentrationen im Drainagewasser lagen während der meisten Zeit im sehr tiefen ng/L Bereich. Die höchste gemessene

Konzentration betrug 35 ng/L. Die kumulativen ZON Mengen, welche übers Drainagewasser vom Feld ausgetragen wurden, bewegten sich während der Wachstumsphase der jeweiligen Kultur zwischen 0.1 und 4.3 mg/ha. Dies entspricht einem Anteil von 0.001 bis 0.070% der anfänglich vorhandenen ZON Menge auf dem Feld.

Unsere Daten zeigen deutlich, dass die Hauptmenge des produzierten ZON in den Boden gelangt. Aufgrund der Verdünnung des Drainagewassers in grösseren Flüssen werden die ohnehin bereits tiefen ZON Konzentrationen unter einen für die Umwelt kritischen Bereich fallen. Allerdings ist wenig bekannt über die ökotoxikologischen Auswirkungen von kurzfristigen Stossbelastungen. In mehreren hundert untersuchten Wasserproben aus verschiedenen Flüssen konnte ZON nur dreimal nachgewiesen werden. In kleineren Flüssen, deren Einzugsgebiet landwirtschaftlich dominiert ist, ist es jedoch denkbar, dass in einem *Fusarium* Jahr ZON zur Gesamtöstrogenizität wesentlich beiträgt.

Während 2 Monaten haben wir neben ZON auch noch das Pilzgift Deoxynivalenol (DON) im Drainagewasser gemessen. Die DON Konzentrationen lagen zwischen 23 ng/L und 4.9 µg/L und waren somit deutlich über den ZON Werten. Ähnlich sah das Bild auch in den grösseren Schweizer Flüssen aus, wo die DON Konzentrationen bis zu 22 ng/L erreichten.

Es ist davon auszugehen, dass von *Fusarium* infizierten Feldern noch weitere Pilzgifte in unsere Oberflächengewässer gelangen können. Einige davon werden in ähnlichen oder grösseren Mengen wie ZON und DON produziert. Daher schlagen wir vor, dass Pilzgifte in Zukunft entsprechend anderen organischen Mikroverun-reinigungen in Gewässern untersucht und beurteilt werden sollten.

Chapter 1: General introduction

1.1 History of endocrine disrupting chemicals

In 1947 – the same year as DDT was introduced - in Florida it was found that the numbers of eaglets began to drop sharply and in the following years weird behaviour in many of the eagle pairs was observed. First it was thought that the large housing development on coastal land was responsible for these observations, but later it was confirmed that around 80% of the bald eagles were sterile. In the late 1950's English hunters observed that the number of otters decreased dramatically or disappeared altogether for no apparent reason. Some suspected the pesticide dieldrin to cause this dramatic decline. In the year 1970 the biologist Gilbertson became witness of a devastating scene on Near Island, lake Ontario. By virtue of his estimation, almost 80% of the gull chicks died in this year before they hatched. Deformities of the dead chicks pointed to the chick endema disease, which was known to be caused by dioxin, at least in lab studies. Surprisingly, neither in the lake water nor in the gull eggs traces of dioxin could be found. In 1980's along the shore of lake Apopka in Florida biologists found that the alligators had no eggs to spare, which could have been used for state's alligator ranching industry. But half of the baby gators that hatched died within a couple days. It was also observed that 60% of the male alligators had abnormally tiny penises. Specialists linked these observations with a chemical accident in 1980 where the pesticide dicofol was spilled into the lake and more than 90% of the alligator population disappeared. But it could not be understood why alligators suffered reproductive problems long after the accident and at a time the lake water was clean again. Then in 1992 a researcher from Copenhagen observed that worldwide average human sperm count dropped by 50% between 1938 and 1990. In the same period cases of testicular cancer increased as well. Because changes happened in such a short period, it was suggested that genetic factors and not environmental factors were responsible. [1]

All the above mentioned observations somehow linked to chemical pollution of the environment, but to this date it was unclear which effects were responsible. Today it is known that the described incidents happened because many of the environmentally present synthetic chemicals have endocrine disrupting properties and are therefore able to interfere with animals and humans sexual development.

[1] This paragraph is summarized from: „Our stolen future", Dutton, Peguin Books (NY), 1996.

Besides chemicals introduced into the environment by human acitivities also sexual abnormalities due to naturally occurring endocrine disrupting chemical were observed. In the 1940's in Australia female sheep, which grazed on pasture with red clover, had difficulties maintaining their pregnancies due to the „clover disease" *(1)*. Over the past 50 years, in Western Australia millions of ewes have been rendered infertile by phyto-estrogens *(2)*.

Besides phytoestrogens also the resorcyclic acid lactones (RALs), in particular zearalenone (ZON) are naturally occurring endocrine disrupting compounds. Owing to their considerable health and economical risk, the occurrence of these mycotoxins has been studied extensively in food and feed products. Agricultural products around the world show contamination rates of ZON as high as 69% of the tested samples with concentrations of up to 180 µg/kg (several studies summarized in Rhyn and Zoller *(3)*). Even larger numbers up to 21 mg/kg were compiled by Pittet et al. *(4)*. A recent overview in Swiss cereal products tested positive for ZON in 13% of all samples at concentrations mostly in the low µg/kg range *(3)*. The authors estimated the mean daily intake of ZON to be < 1 µg/capita/day. This is approximately one order of magnitude lower than the evaluated temporary tolerable daily intake value of the scientific committee on food of the European commission *(5)*. However in general, the results point to a frequent and global occurrence of ZON in food and feed products. Moreover, as the contamination of, e.g., wheat and corn, is most likely not limited to the edible plant organs *(6-8)*, such surveys probably only consider the "tip of the iceberg" of the overall ZON exposure, particularly in the environment.

1.2 NRP50 – Endocrine Disruptors: Relevance to Humans, Animals and Ecosystems

In January 2002 the Swiss national science foundation started the research program number 50 (NRP50) *(9)* "Endocrine Disruptors: Relevance to Humans, Animals and Ecosystems". The NRP50 aimed to develop scientific strategies to assess the risks and hazards that arise when endocrine disruptors are processed through ecosystems to cause human and animal exposure. Methods and models suitable to assess the endocrine activity of these chemicals or chemical mixtures were established and the mechanisms of action, and their effects on developmental and reproductive processes were investigated. The magnitude of exposure of humans, domestic animals, wildlife and environment in Switzerland and the resulting hazards and risks

were assessed. The NRP50 aimed to create a consensus platform for industry and regulators on how to avoid the negative impact of today's chemicals of this category. In the pursuit of this goal, the NRP aimed to define a set of rules for future development of pertinent substances. [2]

In 2003 the idea of an interdisciplinary project grew at Agroscope Reckenholz. For many years, the research group “Ecological plant protection” around Hans-Rudolf Forrer had dealt with the possibilities to reduce *Fusarium* head blight and mycotoxin contamination in organic and IP cereals *(10,11)*. So far the research of mycotoxins was focused on deoxynivalenol (DON). But along with DON other mycotoxins are produced by *Fusarium* spp., such as other trichothecenes, fumonisines, moniliformins and zearalenone (ZON). Most of them are toxic and/or carcinogenic with one exception, the highly estrogenic ZON. So far the problem of ZON contaminated crops was only recognized in the food and feed context. Because *Fusarium* infection and therewith ZON contamination takes place on crop fields and part of the plant material remains on these fields after harvest, a possible ZON input to the environment is plausible. Under these premises, Thomas Bucheli and Hans-Rudolf Forrer submitted the project “Environmental exposure to estrogenic mycotoxins”, which became part of the NRP50. The dissertation born from this project is presented here.

1.3 Introduction to “Environmental exposure to estrogenic mycotoxins”

Mycotoxins are naturally occurring secondary metabolites of fungi growing on a variety of cereals. Among the most important mycotoxin producing field-born fungi are *Fusarium* spp. They pose a severe economical threat, which in the US crop production of the 1990s led to losses of three billion US$ *(12)*. No exact calculations are available for Europe, but in Switzerland both losses of crops and quality have increased in recent years *(13)*. The extent of *Fusarium* infection and subsequent contamination by mycotoxins is determined by several factors such as climatic conditions, crop rotation, soil cultivation and susceptibility of crop varieties *(14)*.

Out of the many classes of mycotoxins produced by *Fusarium* fungi, RALs are of particular concern with respect to endocrine disruption. The most prominent representative of the RALs is ZON. The estrogenic activity of ZON is comparable with those of natural estrogens *(15)* and is orders of magnitudes higher than those of

[2] This paragraph was taken from the NRP50 website: www.nrp50.ch

many notorious synthetic endocrine disruptors such as bisphenol A, DDT or atrazine *(16,17)*. The estrogenically most potent of all RALs, α-zearalanol (α-ZAL), is still used as growth promoter for ruminants in the US and Canada, but has been banned in the EU since 1985. In the past it has been shown that RALs can cause severe reproductive and infertility problems in husbandry animals *(18,19)* due to their high estrogenic potencies.

Although the occurrence of ZON has been studied extensively in food and feed stuff *(3,4)*, only little is known about its environmental distribution and impact. Several publications reported the occurrence of ZON in surface waters *(20,21)* as well as in- and effluents of waster water treatment plants (WWTP) *(20,22,23)*. Concentrations ranged from not detected up to 60 ng/L for individual samples. In some cases also other RALs such as α-zearalenol, α-ZAL and β-zearalanol were detected at similar levels as ZON. For comparison, numbers in the same order of magnitude were observed for natural steroids *(24,25)*. From the limited data summarized in Bucheli et al. *(26)*, RALs seem to appear in surface waters occasionally, and throughout the year. Unfortunately, none of the mentioned studies further investigated on their potential emission sources. Only Lagana et al. *(22)* suspected the presence of RALs in surface waters to be primary caused by cattle excretion of growth promoting residues. RALs data from other environmental compartments are also very limited. Only in one publication *(27)* ZON dissipation in soil was investigated.

1.4 Scope and content of this PhD thesis

The goal of this PhD thesis was to assess the impact and fate of ZON in the environment. Thereby, our main focus was on the ZON input to the terrestrial and aqueous environment from small grain cereal and corn production as this is where mycotoxins are initially produced. To our knowledge, this is the first time the occurrence of ZON and its metabolites were investigated in such detail, and with a view to relate their presence in the environment to possible sources.

In Chapter 2 and 3 the analytical methods developed for ZON quantification in aqueous matrices such as drainage- and surface water, and solid matrices such as soil, manure and sewage sludge are described. In Chapter 4 the distribution of ZON on *Fusarium graminearum* infected wheat and maize fields in plant organs, soil, and its emission via drainage water is described. Chapter 5 deals with the sorption behaviour of ZON in soils and selected model sorbents. In Chapter 6 ZON is

discussed in a broader agro-environmental context, including the field data described in Chapter 4 and ZON levels measured in manure/dung and sewage sludge. In Chapter 7 the focus is widened to other mycotoxins such as the well known deoxynivalenol to relate this work to the general problem of aquatic micropollutants.

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Paper 1

Hartmann, N.; Erbs, M.; Wettstein, F.E.; Schwarzenbach, R.P.; Bucheli, T.D. Quantification of estrogenic mycotoxins at the ng/L level in aqueous environmental samples using deuterated internal standards. *J. Chromatogr. A* **2007**, *1138*, 132-140.

Paper 2

Hartmann, N.; Erbs, M.; Wettstein, F.E.; Hörger, C.; Schwarzenbach, R.P.; Bucheli T.D. Quantification of zearalenone in various solid agro-environmental samples using d6-zearalenone as internal standard. *J. Agric. Food Chem.* **2008**, *in press*.

Paper 3

Hartmann, N.; Erbs, M.; Hörger, C.; Vogelgsang, S.; Forrer, H-R.; Wettstein, F.E.; Schwarzenbach, R.P.; Bucheli T.D. Occurrence of zearalenone on *Fusarium graminearum* infected wheat and maize fields in crop organs, soil and drainage water. *Environ. Sci. Technol.* **2008**, *submitted*.

Paper 4

Hartmann, N.; Sander, M; Wettstein, F.E.; Schwarzenbach, R.P.; Bucheli, T.D. Sorption of zearalenone on soils and selected model sorbents. *In preparation*.

Paper 5

Hartmann, N.; Erbs, M.; Wettstein, F.E.; Hörger, C.; Vogelgsang, S.; Forrer, H-R.; Schwarzenbach, R.P.; Bucheli T.D. Environmental exposure to estrogenic and other myco- & phytotoxins. *Chimia* **2008**, *in press*.

Paper 6

Bucheli, T.D.; Wettstein, F.E.; Hartmann, N.; Erbs, M.; Vogelgsang, S.; Forrer, H.R.; Schwarzenbach, R.P. Fusarium mycotoxins: overlooked aquatic micropollutants? *J. Agric. Food Chem.* **2008**, *56*, 1029-1034.

Chapter 2: Analytical method for aqueous samples

Quantification of Estrogenic Mycotoxins at the ng/L Level in Aqueous Environmental Samples using Deuterated Internal Standards

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Abstract

Because of their pronounced estrogenicity, resorcyclic acid lactones (RALs) are of concern in aqueous environments even at the low ng/L level. Therefore, we developed an accurate, precise and sensitive HPLC-MS/MS method to detect these mycotoxins in different aqueous environmental samples. The compounds investigated included zearalenone, α- and β-zearalenol, zearalanone as well as α- and β-zearalanol. The use of isotope labelled internal standards (in this case deuterated RAL-analogues) ensured an accurate quantification of the target analytes, independent of matrix compounds interfering with the analytes during ionisation and analyte losses occurring during sample preparation. Sample enrichment was carried out by solid phase extraction using Supelclean™ Envi-18 cartridges. Absolute method recoveries for all analytes ranged from 95 to 108%, 70 to 102%, and 76 to 109%, method detection levels from 0.5 to 2.1 ng/L, 0.4 to 1.1 ng/L, and 0.8 to 12.4 ng/L and precision from 3 to 14%, 2 to 13% and 4 to 16% in drainage water, river water and WWTP effluent, respectively. The method was applied to verify the emission of RALs from a *Fusarium graminearum* infested crop field into the drainage system. Zearalenone was present in drainage water in concentrations up to 30 ng/L. So far, none of the other five investigated compounds have been detected.

2.1 Introduction

The relevance of endocrine disruptors in the aqueous environment has been broadly recognized over the past decade *(1)*. Studies on their presence in surface waters have primarily focused on steroid hormones such as 17-β-estradiol and estrone as well as synthetic compounds like nonylphenol, bisphenol A, 17-β-trenbolone, chlorinated pesticides such as lindane, and herbicides like diuron *(1,2)*. Less attention has been paid to naturally occurring estrogenic myco- and phytotoxins. Resorcyclic acid lactones (RALs) (Figure 2.1) have been of concern particularly as contaminants in food and feed *(3)* due to their very high relative estrogen receptor binding affinities and estrogenic potencies *(4-6)*. The RALs are produced by *Fusarium graminearum* fungi, which colonise a wide variety of crops like wheat, barley, oat and corn *(7).* The amount of *Fusarium* fungi infested crops has increased worldwide during the last 10-15 years *(8).* It is currently believed that this is caused by altered cultivation techniques, such as the increasing use of soil conservation rather than plough tillage, simplified crop rotation with a high fraction of cereals and corn, as well as climate changes *(9-11).*

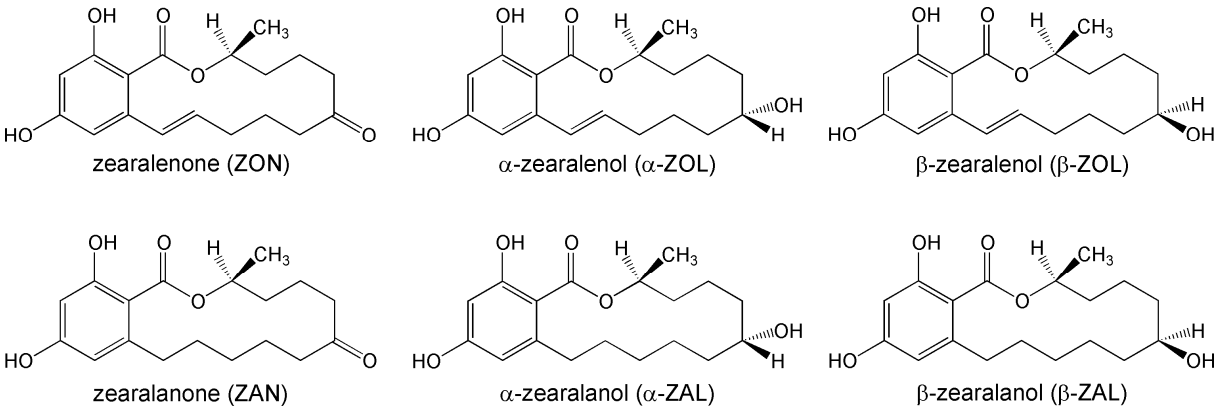

Figure 2.1: Chemical structure and acronyms of the resorcyclic acid lactones (RALs)

The occurrence of RALs in surface waters and wastewater treatment plant (WWTP) effluents was recently reported (for an overview, see Bucheli et al. 2005 *(12)*). Concentrations up to 60 ng/L were reported for zearalenone (ZON) and some of its metabolites in these waters. We hypothesized that agricultural fields are potential sources of RALs and that these estrogenic mycotoxins enter surface waters via drainage water and surface runoff *(12).* To accurately and precisely quantify the

expected low concentrations of RALs in these aqueous samples, a very selective and sensitive analytical method is required.

For this purpose, some studies resorted to GC-MS *(13,14).* However, HPLC-MS/MS is the state of the art technique for polar organic micropollutants, such as the RALs *(15,16).* The crucial step in HPLC-MS/MS is the ionisation of the analyte, which may be affected by competing sample matrix components such as dissolved organic matter *(17-19).* The resulting ion suppression or enhancement has a profound influence on accuracy, precision and sensitivity when it comes to trace level quantification of pollutants in environmental samples. It is usually compensated by matrix matched calibration *(21,22)* or standard addition *(22,23)* but these methods are hardly applicable for analysis in dynamic environmental systems with fast changing matrices (e.g. river water). Alternatively, co-extraction of unwanted matrix components can be efficiently avoided by using highly selective immuno-affinity columns containing antibodies targeting only the analytes desired. Several such columns are actually available for ZON and are currently tested in our laboratory for their cross-reactivities with other RALs *(24)*. However, their high costs prevent them from being applied for extraction or clean-up in routine monitoring studies. Despite the fact that ion suppression is a well-known problem, none of the currently reported HPLC-MS/MS methods for RALs have investigated ion suppression quantitatively *(13,15,16).* The use of isotope labelled internal standards is the powerful way of compensating matrix effects during the ionisation process in the ion source of the MS/MS instrument *(25)* as well as losses during sample preparation, thus ensuring accurate and precise results. If deuterated internal standards (DIS) are available, their application is a very time-effective alternative to standard addition or matrix matched calibration. Although deuterated RALs have been used in different excretion studies *(26,27),* they have not yet been applied as internal standards in environmental trace analysis. Hence, one important objective in this work is to demonstrate that DIS are crucial for accurate and precise quantification of the RALs in environmental samples.

In this study, we describe an accurate, precise and sensitive HPLC- negative electrospray ionisation ($^{-}$ESI)-MS/MS analytical method for RALs in various aqueous samples. The method comprises solid phase extraction for enrichment of six prevalent RALs and purification of the samples. To our knowledge, this is the first time DIS are used for quantifying these six RALs in different aqueous environmental

samples. Furthermore, the analytical method presented is validated for RALs in Milli-Q water, drainage water, river water and WWTP effluent, and its application is demonstrated in a field study on the runoff of these estrogenic mycotoxins from agricultural fields.

2.2 Experimental section

2.2.1 Chemicals

ZON [17924-92-4] (≥99%), zearalanone [42422-68-4] (ZAN) (≥98%), α-zearalanol [5975-78-0] (α-ZAL) (≥97%), β-zearalanol [26538-44-3] (β-ZAL) (≥98%), α-zearalenol [36455-72-8] (α-ZOL) (≥98%) and β-zearalenol [71030-11-0] (β-ZOL) (≥95%) were purchased from Sigma (Buchs, Switzerland). For structures, see Figure 2.1. The DIS D_4-α-ZAL (purity n.a.), D_4-β-ZAL (purity n.a.), D_4-α-ZOL (≥95%) and D_4-β-ZOL (≥95%) were purchased from RIVM (Bilthoven, The Netherlands). D_6-ZON was prepared in our own laboratory by base-catalyzed hydrogen-deuterium exchange on native ZON as described in Miles et al. 1996 *(28).* According to Miles et al. *(28)* hydrogen-deuterium exchange takes place at the positions C-3, C-5, C-5' and C-7'. The purity of the internal standard for ZON (D_6-ZON) was tested by scanning the masses of ZON and D_n-ZON (n=1-9). The relative amounts were 0.16% 0.10%, 0.83%, 6.16%, 25.07%, 38.32%, 23.13%, 5.50%, 0.70% and 0.03% for ZON, D_1-ZON, D_2-ZON, D_3-ZON, D_4-ZON, D_5-ZON, D_6-ZON, D_7-ZON, D_8-ZON and D_9-ZON, respectively. Ratios of the different grades of deuterated ZON's did not change over time. Therefore, D_6-ZON was found to be a suitable internal standard and the other deuterated ZON products did not influence quantification. Methanol (MeOH, 99.98%) and acetonitrile (ACN, 99.99%) were purchased from Scharlau (Barcelona, Spain). $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 2H_2O$ (both p.a. quality) were obtained from Merck AG (Dietikon, Switzerland) and ammonium acetate was purchased from Fluka AG (Buchs, Switzerland). Deionised water was produced by a Milli-Q gradient A10 water purification system from Millipore (Volketswil, Switzerland). N_2 (99.99995%) was purchased from PanGas (Dagmarsellen, Switzerland).

Individual stock solutions holding concentrations of 500 mg/L were prepared in pure MeOH for all analytes. Multicomponent stock solutions were prepared in MeOH in concentrations of 10, 100, 1'000 and 10'000 ng/mL for each constituent. The internal standard solution was prepared in MeOH and had a concentration of 2 μg/mL for all

five DIS. Since no DIS is commercially available for ZAN and no synthesis procedure for ZAN deuteration could be found in the literature, D_6-ZON was used as an internal standard for the quantification of both ZAN and ZON. Aqueous calibration standards holding all six RALs equivalent to the concentration range of 0.5-100 ng/L, and 100 ng/L of all five DIS were prepared in Milli-Q water from the methanolic multicomponent stock solutions. The stability of the DIS solution in MeOH was tested over several months. No deuterium-hydrogen exchange was observed within this time period. All RALs were stable when present in single- and multicomponent methanolic stock solutions. All stock solutions were stored at -20℃.

2.2.2 Sample collection and preparation

Drainage water samples were collected at our field study site at Reckenholz, Switzerland *(12)* using portable automatic flow-proportional samplers (Teledyne Isco Inc., Lincoln NE, USA.). Surface water samples were collected at various sampling sites throughout Switzerland maintained by monitoring programs of the Swiss government (National Long-Term Surveillance of Swiss Rivers; NADUF) and the Canton of Zürich (Office for Waste, Water, Energy, and Air; AWEL). WWTP effluent samples were collected at the Zürich-Werdhölzli (Zürich, Switzerland) facility. Raw water samples were filtered (glass fibre filters, pore size 1.2 μm, Millipore, Volketswil, Switzerland) by vacuum filtration (Supelco, Bellfonte PA, USA), transferred to 1 L glass bottles and stored in the dark at 4 ºC until analysis within two weeks. Control experiments with spiked samples revealed a less than 10% concentration change of all RALs in all matrices within two weeks. The exact volume of 1 L was spiked with 50 μL of the internal standard solution. Samples were shaken vigorously before further treatment.

2.2.3 Solid phase extraction (SPE)

Filtered water samples were concentrated and purified by performing reversed-phase SPE (Supelclean™ Envi-18 SPE cartridges, 6 mL, 500 mg, Supelco, Bellfonte PA, USA). The SPE cartridges were conditioned with 5 mL of ACN and 4 mL of Milli-Q water consecutively. Water samples were drawn through the cartridges at a flow rate of max. 10 mL/min and subsequently air-dried for at least 10 min. No washing step was performed in order to avoid analyte losses. The analytes were eluted with 4 mL

ACN. The eluates were evaporated to dryness using a gentle nitrogen gas stream at 50 ºC for approx. 30 min. The dried extracts were reconstituted in 300 µL of Milli-Q water/ACN (80/20, v/v) and transferred into 350 µL amber glass vials. The samples were stored at 4 ℃ and analysed within 48 hours.

2.2.4 LC-MS/MS analysis

LC-MS/MS was performed on a Varian 1200L LC-MS instrument (VarianInc, Walnut Creek, CA). The RALs were separated on a Polaris Amide-C18 column (150 x 2.0 mm, 3 µm, VarianInc) at room temperature by applying the following elution gradient: 0 min 0% B (100% A), 3min 0% B, 4 min 40% B, 25.5 min 67.5% B, 26 min 100% B, 29 min 100% B, 30 min 0% B, 35 min 0% B; with eluent A consisting of Milli-Q water/ACN (95/5, v/v) and eluent B of Milli-Q water/ACN (5/95, v/v). Both eluents were buffered with 10 mM ammonium acetate (pH=6.8). The injection volume was 50 µL and the mobile phase flow rate was 0.2 mL/min. Post-column addition of 0.3 mL/min MeOH enhanced signal intensity by a factor of 1.5. Interface parameters of the LC-MS/MS were as follows: needle voltage -2000 V, nebulizing gas (compressed air) 4.21 bar, capillary voltage -54 V, drying gas (N_2, 99.5%) 300 ºC and 1.59 bar, shield voltage -600 V. Detection of the RALs was performed in the (-)ESI mode using the mass transition reactions specified in Table 2.1. The collision cell gas (Ar, 99.999%) pressure was 2.0 e^{-6} Torr and the detector voltage was set to 2000 V. Data acquisition was divided into three retention time segments: segment 1: β-ZAL and β-ZOL, and respective DIS, segment 2: α-ZAL and α-ZOL, and respective DIS, and segment 3: ZAN, D_6-ZON and ZON (see Figure 2.2). Retention times for the RALs and the DIS are given in Table 2.1. The analytes were quantified using external calibration, i.e. calibration standards in Milli-Q water containing the five DIS (see above). Data processing was carried out using the software Varian MS Workstation (v. 6.5).

Table 2.1) Analytical parameters for the RALs and their corresponding deuterated internal standards

Substances	Retention time	Precursor ion	Main product ion		Secondary product ion	
	min	m/z	m/z	Collision Energy (eV)	m/z	Collision Energy (eV)
D_4-β-zearalanol	19.04	325.4	281.0	20	263.0	25
β-zearalanol	19.12	321.4	277.0	15	303.0	20
D_4-β-zearalenol	19.53	323.4	279.0, 174.0[a]	20, 30[a]	174.0, 160.0[a]	30, 25[a]
β-zearalenol	19.65	319.4	275.0	20	160.0	30
D_4-α-zearalanol	22.18	325.4	281.0, 163.0[a]	25, 20[a]	163.0, 192.0[a]	20, 30[a]
α-zearalanol	22.31	321.4	277.0	15	259.0	25
D_4-α-zearalenol	23.40	323.4	280.0	25	133.0	30
α-zearalenol	23.43	319.4	275.0	20	205.0	25
zearalanone[b]	25.43	319.4	275.0	20	205.0	25
D_6-zearalenone	25.93	323.4	134.0	30	279.0	20
zearalenone	26.09	317.4	131.0	26	175.0	20

[a] Product ions used for river water and WWTP effluent.
[b] No corresponding DIS. Instead D_6-zearalenone was used as internal standard.

2.2.5 Ion suppression

Matrix effects were evaluated by comparing the analyte signals obtained from injection of the same amount of analyte dissolved in pure solvent and an extracted matrix blank (both in Milli-Q water/ACN (80:20)). For this purpose, 24 L of each matrix (drainage water, river water and WWTP effluent) were filtered and subjected to SPE as described above. The 24 SPE eluates were combined and divided into eight equal portions. 50 µL of internal standard solution was added to each portion. Standard addition to the matrix extraction eluates was carried out in order to yield concentrations equivalent to 1 L samples containing 0.5, 5, 10, 20, 40, 60, 80 and 100 ng/L of each analyte. Curves were obtained by plotting measured analyte peak areas against corresponding analyte concentration levels in pure solvent and in extracted matrix blank, respectively. Linear regression was performed for each curve. The ion suppression was quantified as the ratio between the slope of the curve for the pure solvent and the slope of the curve obtained for the extracted matrix blank.

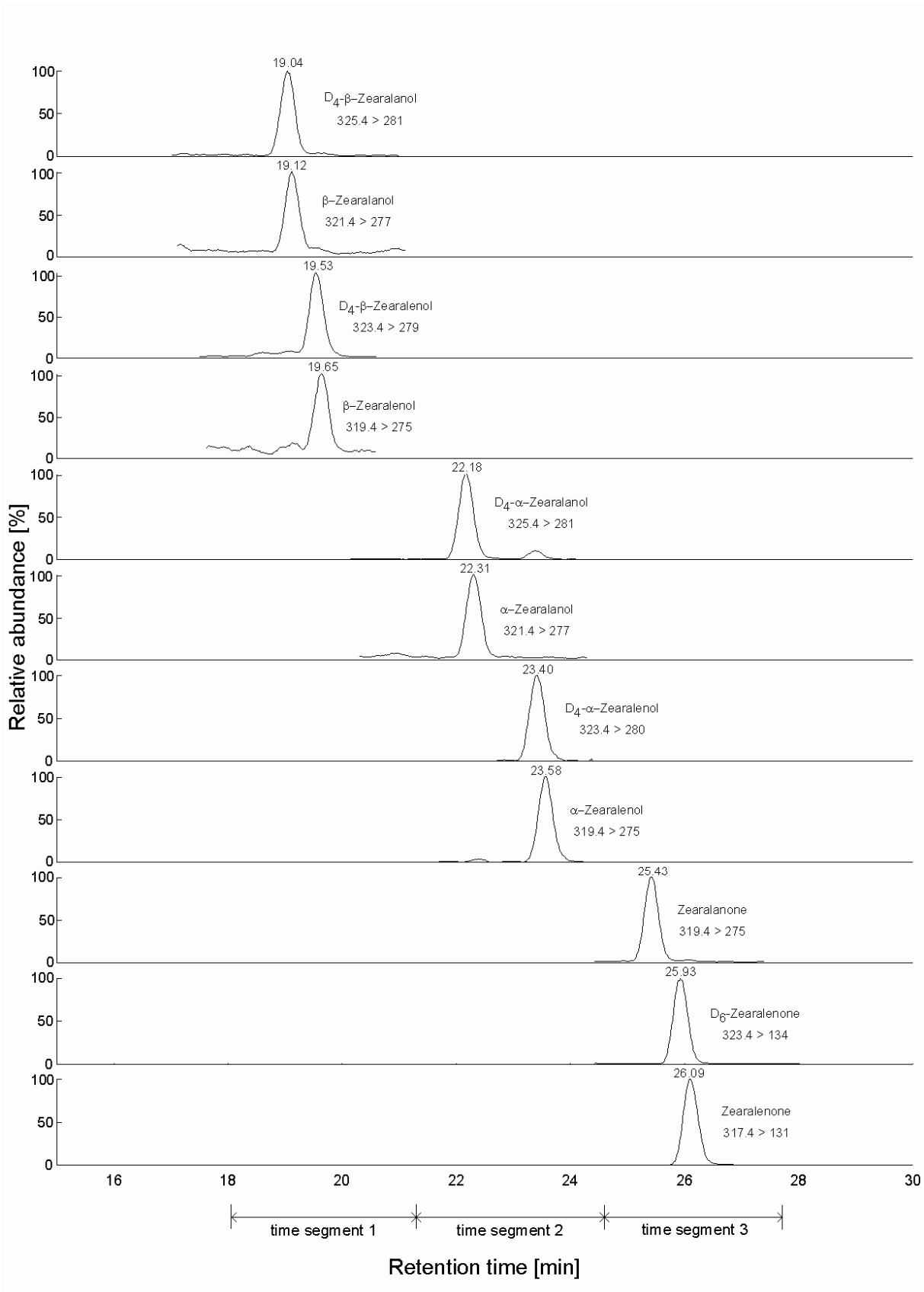

Figure 2.2: LC-MS/MS chromatograms from drainage water extracts spiked with 25 ng/L of each of the investigated resorcyclic acid lactones (RALs). For details on the instrumental parameters, see text and Table 2.1.

2.2.6 Absolute and relative recoveries, precision, method detection limit (MDL) and linearity

Absolute recoveries were obtained for all RALs in Milli-Q water, drainage water, river water and WWTP effluent. One liter samples were spiked with RALs to yield three concentration levels: 5, 25 and 100 ng/L. One liter of unfortified matrix, i.e. containing DIS only, was tested for native RAL content and/or contamination due to the addition of DIS. Five replicates were prepared for every concentration level. The samples were processed and quantified as described in sections 2.2.2-2.2.4. Sample residues were reconstituted in 250 μL Milli-Q water/ACN (80/20, v/v) and 50 μL internal standard solution. The absolute method recovery was defined as the ratio between the quantified and the spiked amount.

Relative method recoveries were determined for the matrices mentioned above. Samples were processed at the same three concentration levels each with five replicates as described above, including internal standard solution added prior to SPE resulting in a concentration of 100 ng/L. The relative recovery was defined as the ratio between the quantified and the spiked amount. The precision of the analytical method was defined as the mean relative standard deviation of five replicates at the chosen concentration levels.

For all analytes, the MDL was defined as three times the mean absolute standard deviation of the five replicates at the concentration level of 5 ng/L *(29).* For ZON, which had a blank level of 0.16% originating from the internal standard solution, the MDL was also calculated as three times the mean blank level. The higher of the two values calculated for ZON was set as the MDL. The linearity of the MS/MS detector was tested with MilliQ water containing RALs at concentrations between 0.1 ng/L and 100 ug/L, corresponding to 5 pg to 5 ug at the detector.

2.3 Results and discussion

2.3.1 Chromatographic separation and MS detection

Since some of the analytes have the same molecular mass and formed the same product ions, baseline separation was required to distinguish between them. This is shown in Figure 2.2. For all analytes, including DIS, deprotonated molecules $[M-H]^-$ were the major precursor ions formed and two product ions were monitored for each analyte. The most abundant product ion was used for quantification and a second

product ion for confirming analyte identity (see Table 2.1). Due to compounds co-eluting with D_4-β-ZOL and D_4-α-ZAL in river water and WWTP effluent extracts, more selective but less intensive product ions than those probably resulting from neutral losses (elimination of CO_2, m/z = 44) had to be chosen. Collision energies were optimised for each analyte individually (see Table 2.1).

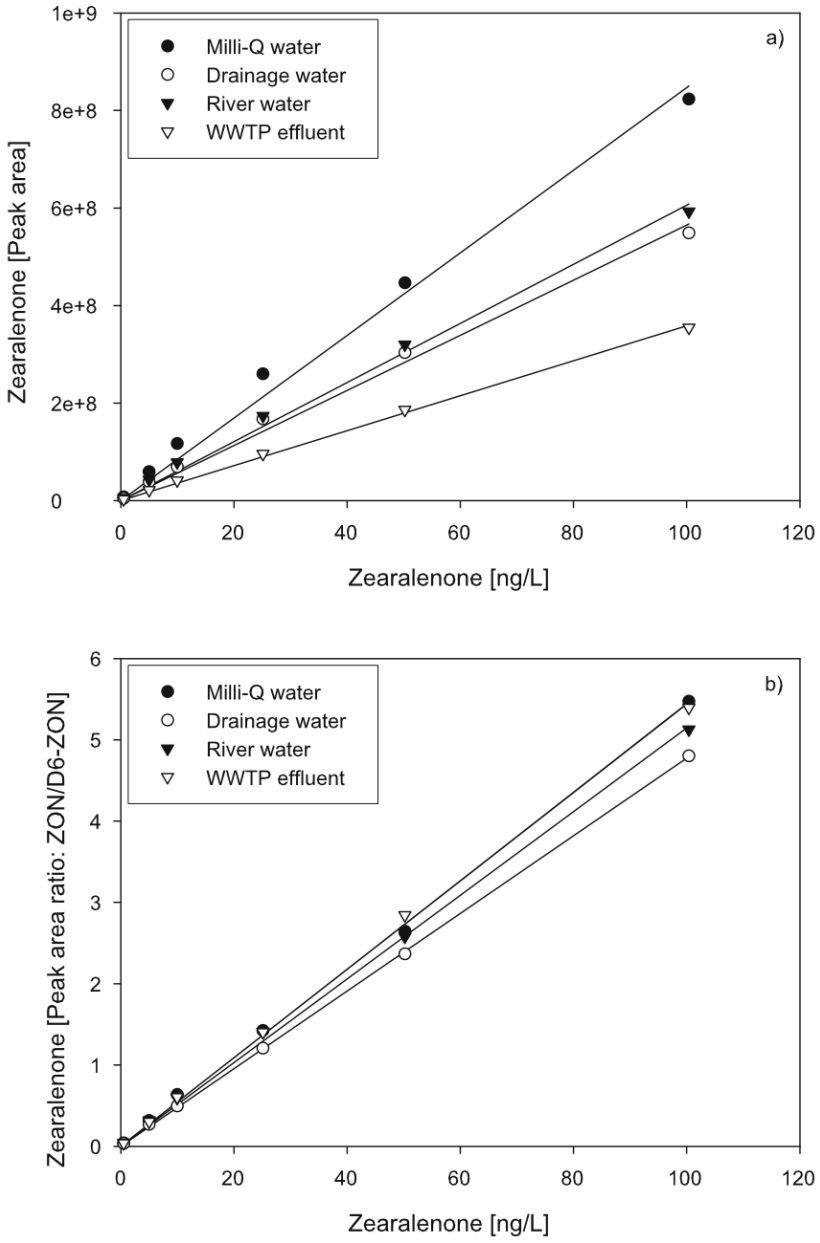

Figure 2.3: (a) Measured Zearalenone (ZON) peak area versus ZON concentration in Milli-Q water (●) and in matrix eluates (drainage water (○), river water (▼), WWTP effluent (□)). The reduced slopes obtained for environmental extracts visualize the ion suppression effect. (b) Ratio of ZON peak area to D6-ZON peak area versus concentration in Milli-Q water (●) and in matrix eluates (drainage water (○), river water (▼), WWTP effluent (□)). Ion suppression is effectively compensated for by normalization to DIS.

2.3.2 Ion suppression during ionization in the ion source

The extent of ion suppression was quantified for all analytes in drainage water, river water and WWTP effluent as the ratio between the slopes of calibration curves obtained in the respective matrix and the one in Milli-Q water. Such curves are shown for ZON in Figure 2.3a, where ion suppression varying from 28% in river water to 58% in WWTP effluent can be seen. Ion suppression (28-68%) occurred for all analytes in all investigated matrices (see Table 2.2), and generally depend on the type of matrix. Ion suppression was consistently higher in WWTP effluent than in drainage and river water. This is plausible considering the high output of organic material from WWTPs. Moreover, ion suppression varied with retention time, i.e. co-extracted interferences had a greater influence on the signal response of β-ZAL and β-ZOL (time segment 1) than on α-ZAL and α-ZOL (time segment 2) and ZAN and ZON (time segment 3). Unfortunately, there are no other ion suppression data available in the literature for RALs in comparable matrices. However, though care must be taken, it is interesting to compare our data with such of other compound classes holding properties similar to the RALs. For instance, Freitas et al. *(25)* reported ion suppression values up to 71% for a variety of equally hydrophobic pesticides in surface waters.

Table 2.2) Ion suppression of resorcyclic acid lactones in various natural water extracts

Substances	Drainage water (%)	River water (%)	WWTP effluent (%)
β-zearalanol	57	49	66
β-zearalenol	57	54	68
α-zearalanol	45	32	57
α-zearalenol	43	38	67
zearalanone	35	28	56
zearalenone	33	28	58

2.3.3 Absolute method recoveries

The absolute recoveries determined for all RALs in Milli-Q water, drainage water, river water and WWTP effluent at three different concentration levels are summarised in Table 2.3. As the pH values and the ionic strength vary for the investigated matrices, the influence of these parameters on the absolute recovery of the RALs was examined in Milli-Q water first. Measured pH values were 6.3 for Milli-Q water, 6.8 for drainage water, 8.3 for river water and 7.3 for WWTP effluent. The ionic strengths were not measured but were expected to be in the range 10-20 mM for

drainage water, river water and WWTP effluent *(30).* Hence, Milli-Q water was adjusted to pH=5.0, 7.0 and 8.5, by means of a phosphate buffer (10 mM P (NaH_2PO_4/Na_2HPO_4)). For ZON (at 5 ng/L), the absolute recovery in Milli-Q water increased from 76% (unbuffered, pH=6.3) to 93% (pH=5), 90% (pH=7) and 90% (pH=8.5), respectively, when the phosphate buffer was added. Similar increases were observed for the other RALs (see Table 2.3). The results acquired indicate that the presence of 10 mM P in Milli-Q water increased absolute recoveries for all RALs whereas an increase in pH did not influence the recovery rates significantly which is not surprising due to the estimated pK_a values. Acidity constants (pK_a) have been reported only for α-ZAL (pK_{a1} = 8.44, pK_{a2} = 11.42) (31). However, when considering the very similar chemical structures of the RALs, it is reasonable to assume that the dissociation constants for all RALs lie in the range 8.5-11.5. The pK values for resorcinol (1,3-dihydroxybenzene), which equals the part of the RALs containing the functional groups, compare with these values as well (pK_{a1} = 8.62, pK_{a2} = 11.07; *(32)*).

Table 2.3) Absolute recoveries of resorcyclic acid lactones at low concentrations in various natural waters[a]

Substances	Conc (ng/L)	Milli-Q-water (%)				Drainage water (%)	River water (%)	WWTP effluent (%)
		Buffered[b] pH=5.0	Buffered[b] pH=7.0	Buffered[b] pH=8.5	Unbuffered pH=6.3	pH=6.8	pH=8.3	pH=7.3
β-zearalanol	5	103 (8)	98 (10)	106 (12)	68 (6)	108 (7)	100 (6)	n.a.
	25	n.m.	n.m.	n.m.	83 (8)	106 (5)	101 (2)	84 (4)
	100	n.m.	n.m.	n.m.	90 (4)	103 (4)	104 (2)	88 (4)
β-zearalenol	5	89 (3)	85 (6)	84 (3)	70 (7)	97 (5)	90 (15)	n.a.
	25	n.m.	n.m.	n.m.	65 (9)	82 (5)	99 (8)	86 (8)
	100	n.m.	n.m.	n.m.	73 (4)	84 (6)	97 (6)	93 (1)
α-zearalanol	5	83 (7)	76 (6)	80 (6)	71 (7)	101 (3)	102 (14)	n.a.
	25	n.m.	n.m.	n.m.	85 (7)	107 (7)	99 (11)	99 (5)
	100	n.m.	n.m.	n.m.	85 (2)	102 (1)	98 (10)	92 (8)
α-zearalenol	5	100 (12)	89 (9)	95 (7)	86 (5)	101 (3)	70 (4)	n.a.
	25	n.m.	n.m.	n.m.	83 (13)	110 (14)	95 (10)	98 (12)
	100	n.m.	n.m.	n.m.	88 (3)	104 (5)	92 (11)	94 (6)
zearalanone	5	84 (3)	88 (1)	88 (6)	75 (8)	95 (6)	92 (7)	n.a.
	25	n.m.	n.m.	n.m.	85 (10)	111 (4)	102 (5)	88 (6)
	100	n.m.	n.m.	n.m.	87 (2)	100 (4)	100 (6)	88 (2)
zearalenone	5	93 (5)	90 (1)	90 (5)	76 (7)	104 (5)	91 (4)	109 (10)
	25	n.m.	n.m.	n.m.	78 (14)	111 (3)	97 (7)	98 (8)
	100	n.m.	n.m.	n.m.	82 (3)	103 (1)	98 (6)	94 (3)

[a] Absolute standard deviation (five replicates) in brackets.
[b] Buffered using 10 mM P (NaH_2PO_4/Na_2HPO_4).
n.m.: not measured.
n.a.: not available due to higher method detection limit.

In natural waters, absolute recoveries ranged from 95 to 108%, 70 to 102%, and 76 to 109% for drainage water (5 ng/L), river water (5 ng/L) and WWTP effluent (25 ng/L), respectively. At higher concentration levels (25 and 100 ng/L), the recovery rates were in the same range for all RALs. Thus, absolute recoveries did not seem to depend on analyte concentration – at least not in the concentration range investigated (see Table 2.3).

2.3.4 Relative method recoveries

We applied DIS to obviate the highly variable and unpredictable matrix effects that affect the SPE, the concentration step and the ionisation of our analytes. Ideally, an internal standard should have the same SPE affinity and be affected by co-extracted and -eluting matrix constituents to a similar extent as the target analyte. Hence, it should have the same physical and chemical properties. In the case of RALs, this applies to their respective deuterated analogues. Relative recoveries close to 100% (Table 2.4) indicate that the DIS were able to compensate losses from the SPE to the ionisation and thus were suitable as internal standards. As seen in Figure 2.3b, the ratios of ZON to D_6-ZON peak areas are constant within the analyte concentration range and identical for all matrices. This holds for all analyte/DIS ratios in all matrices and basically legitimates the use of DIS fortified calibration standards in Milli-Q water. Overall, our findings demonstrate that applying DIS is an effective way of compensating analyte losses during extraction and analyte signal suppression caused by matrix effects.

Table 2.4) Relative recoveries[a] and precision of resorcyclic acid lactones at low concentrations in various natural waters

Substances	Conc. (ng/L)	Milli-Q-water pH=6.3, unbuffered		Drainage water pH=6.8		River water pH=8.3		WWTP effluent pH=7.3	
		Rel. rec. (%)	Prec. (%)	Rel. rec. (%)	Prec. (%)	Rel. rec. (%)	Prec. (%)	Rel. rec. (%)	Prec. (%)
β-zearalanol	5	121 (10)	8	112 (6)	3	105 (6)	6	n.a.	n.a.
	25	108 (3)	3	106 (7)	4	112 (3)	2	99 (9)	9
	100	107 (3)	3	99 (9)	6	109 (3)	3	90 (10)	11
β-zearalenol	5	113 (6)	6	111 (7)	9	101 (7)	7	n.a.	n.a.
	25	105 (2)	2	96 (8)	6	104 (6)	6	103 (7)	7
	100	102 (6)	6	90 (6)	3	106 (5)	4	94 (6)	6
α-zearalanol	5	109 (7)	6	112 (13)	10	100 (3)	3	n.a.	n.a.
	25	117 (4)	3	109 (6)	4	89 (12)	13	113 (18)	16
	100	112 (3)	3	100 (7)	3	89 (10)	11	121 (15)	12
α-zearalenol	5	114 (10)	9	91 (13)	12	109 (8)	7	n.a.	n.a.
	25	119 (9)	7	110 (8)	5	99 (5)	5	118 (7)	6
	100	107 (10)	9	103 (11)	8	105 (8)	8	98 (12)	12
zearalanone	5	108 (9)	9	118 (19)	14	101 (4)	4	n.a.	n.a.
	25	121 (4)	3	118 (10)	5	104 (4)	4	102 (5)	5
	100	106 (4)	4	103 (8)	5	99 (5)	5	101 (6)	6
zearalenone	5	112 (5)	4	107 (8)	4	113 (5)	5	115 (6)	5
	25	115 (4)	3	114 (7)	3	114 (5)	5	108 (6)	5
	100	108 (2)	2	102 (8)	5	106 (4)	3	104 (4)	4

[a] Absolute standard deviation (five replicates) in brackets.
n.a.: not available due to higher method detection limit.

2.3.5 Precision, blank levels and method detection limits, repeatability, linearity

The precision (see Table 2.4) of the analytical procedure was verified by multiple analyses of spiked matrix samples. The relative standard deviations (RSD) were calculated at three concentration levels (5, 25 and 100 ng/L) from five replicates of each matrix. Their values ranged from 4 to 9% for Milli-Q water, 3 to 14% for drainage water and 3 to 7% for river water at a concentration level of 5 ng/L. Due to the higher MDL (see below) for WWTP effluent, the RSD was calculated at a concentration level of 25 ng/L. These values ranged from 5 to 16%. The precision obtained here lies well within the commonly reported range *(25)* and confirms the robustness of the presented method. Similar precision numbers were achieved without using DIS *(13,15,16).*

The MDL's for all RALs in all matrices are given in Table 2.5. Two different approaches were chosen for quantifying the MDL. First, for all analytes the MDL was defined as three times the absolute standard deviation of five replicates at 5 ng/L (Milli-Q, drainage, and river water), and 25 ng/L (WWTP effluent). Second, the D_6-ZON (internal standard for ZON and ZAN) contains itself 0.16% of ZON. Therefore,

the MDL for ZON was also calculated by multiplying the blank level (0.16 ng/L) by three as described above. For ZON the higher of the two calculated values was set as the MDL. The values range from 0.7 to 1.4 ng/L in Milli-Q water, 0.5 to 2.1 ng/L in drainage water, 0.4 to 1.1 ng/L in river water and 0.8 to 12.4 ng/L in WWTP effluent. The MDL's obtained here are comparable to the MDL's achieved in earlier published methods for the determination of RALs in aqueous environmental samples *(13,15,16)* as well as for other environmentally relevant compounds like pesticides *(25).*

Instrument variation was assessed for a time period of three months. During this time the same sample in Milli-Q water was quantified eight times. The thus obtained instrument repeatability (RSD) was between 5 and 8% for all RALs. The MS/MS detector was linear between 5 pg and 50 ng, corresponding to 0.1 and 1000 ng/L.

Although the figures of merits presented here are comparable to what has been published for analytical methods that did not rely on *(15,16),* it is conceivable that the accuracy of our method is superior. When matrix effects are compensated by the use of DIS, the results quantified from environmental samples are expected to be closer to the "true" values than when using a non-labelled analogue as an internal standard.

Table 2.5) Method detection limit (MDL)[a] of resorcyclic acid lactones at low concentrations in various natural waters

Substances	Milli-Q-water (ng/L)	Drainage water (ng/L)	River water (ng/L)	WWTP effluent (ng/L)
β-zearalanol	1.4	0.5	0.9	6.3[b]
β-zearalenol	0.9	1.8	0.9	4.8[b]
α-zearalanol	1.0	1.5	0.4	12.4[b]
α-zearalenol	1.4	1.4	1.1	2.3[b]
zearalanone	1.3	2.1	0.6	3.7[b]
zearalenone	0.7	0.5[c]	0.7	0.8

[a] Three times the absolute standard deviation at 5 ng/L.
[b] Three times the absolute standard deviation at 25 ng/L.
[c] Three times the blank level.

2.3.6 Environmental application

We are currently using the analytical method presented here for studying the emission of RALs from a drained field cultivated with winter wheat at Reckenholz, Zürich (Switzerland). The wheat was artificially infected with different ZON-producing *Fusarium graminearum* strains in June 2005 and flow-proportional drainage water samples have been collected regularly prior to and following this infestation. At present, of the investigated RALs, only ZON has been detected in drainage water samples from this field site. Precipitation, water discharge and the resulting ZON

concentration gradient in the drainage water are shown for a rain event in July 2005 (Figure 2.4).

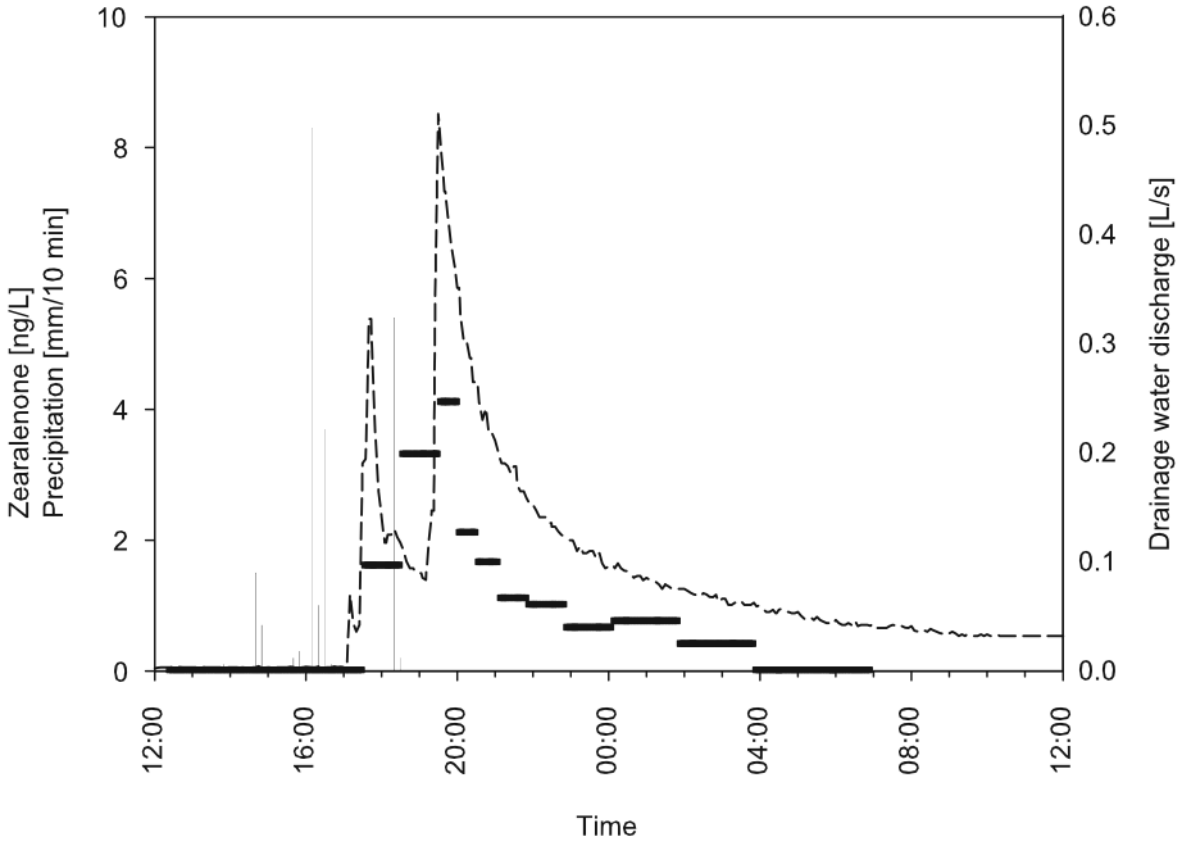

Figure 2.4: Precipitation (|), drainage water discharge (┈) and the resulting Zearalenone concentration (━) in the drainage water during a precipitation event in July 25-26, 2005.

The water discharge in the drainage system started up within the initial hours of rainfall and a close correlation between discharge and drainage water ZON concentration is discernable. This correlation indicates a fast elution of ZON from the wheat plants through the soil into the drainage water presumably due to preferential flow. Detailed results from the currently ongoing field studies mentioned above will be published elsewhere. The fact that the ZON concentration dynamics (Figure 2.4) is exhibiting consistency and agro-environmental chemical plausibility in the concentration range of a few ng/L renders further credibility to the here presented method.

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Chapter 3: Analytical method for solid samples

Quantification of Zearalenone in Various Solid Agro-Environmental Samples Using D_6-Zearalenone as Internal Standard

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Abstract

Because of its pronounced estrogenicity, zearalenone may be of concern not only in the aqueous, but also in the terrestrial environment. Therefore, we developed several analytical methods to quantify zearalenone in different solid matrices of agro-environmental relevance, i.e., plant organs, soil, manure and sewage sludge. The use of D_6-zearalenone as internal standard (IS) was essential to render the analytical method largely matrix-independent, because it compensated target analyte losses during extract treatment and ion suppression during ionization. Soil and sewage sludge samples were extracted with Soxhlet, whereas plant material and manure samples were extracted by liquid solvent extraction at room temperature. Absolute recoveries for zearalenone were 70 to 104% for plant materials, 105% for soil, 76% for manure and 30% for sewage sludge. Relative recoveries ranged from 86 to 113% for all matrices, indicating that the IS was capable to largely compensate losses during analysis. Ion suppression between 8% and 74% was in all cases compensated by the IS, but influenced method quantification levels. These were 3.2 to 26.2 ng/$g_{dry\ weight\ (dw)}$ for plant materials, 0.7 ng/g_{dw} for soil, 12.3 ng/g_{dw} for manure and 6.8 ng/g_{dw} for sewage sludge. Plant material concentrations varied from 86 ng/g_{dw} to more than 16.7 µg/g_{dw}, depending on the organ and crop. Soil concentrations were between not detectable and 7.5 ng/g_{dw}, depending on the sampling depth. Zearalenone could be quantified in all manure samples in concentrations between 8 and 333 ng/g_{dw}. Except for two of 85 investigated sewage sludge samples, zearalenone concentrations were below quantification limit.

3.1 Introduction

In the past, natural and anthropogenic endocrine disrupting chemicals (EDC) have mainly been studied in the aqueous environment *(1)*. However, the aqueous fauna is not the sole group of organisms that is exposed to EDC. Soil organisms such as rodents and husbandry animals *(2)* are also potentially affected by such micropollutants. Sewage sludge contains EDC *(3,4)* and is used for application on agricultural land as fertilizer in many countries of the European Union and the US *(5)*. Animal excretion and application of manure on grassland fields are additional input sources of EDC to the terrestrial environment *(6)*. Depending on the compound, other sources such as atmospheric deposition or compost application also add to the total loads of EDC to soil *(7)*.

Less attention has been paid to naturally occurring estrogenic toxins such as the resorcyclic acid lactones *(3,8)*. Zearalenone, the main representative of the resorcyclic acid lactones, is produced by *Fusarium* species, which colonise a wide variety of crops like wheat, corn, barley or oat *(9)*. Zearalenone is of particular concern due to its very high relative estrogen receptor affinity and estrogenic potency *(10,11)*. Its levels in animal feed and pet products reached up to several µg/g *(12,13)*. Ingested zearalenone is either excreted directly or metabolized and then excreted via urine and/or faeces *(14-17)*. So far zearalenone contents in sewage sludge have not been investigated. Because of the occurrence of zearalenone in wastewater treatment plant (WWTP) in- and effluents *(18,19)* and its affinity to organic carbon (data from own experiments), it is expected to be present also in sewage sludge. Hence, besides *Fusarium* infested crop fields *(20)* manure and sewage sludge could be other potential input sources for zearalenone to the agricultural environment, especially to soil.

To accurately and precisely quantify the amounts of zearalenone expected to reach agricultural soils via *Fusarium* infested crops, manure and sewage sludge, sensitive and robust analytical methods are required. Several methods for quantification of zearalenone in wheat were published *(21-23)*. Cramer et al. *(24)* recently described a method quantifying zearalenone in cereal products using D_2-zearalenone as internal standard (IS). Analytical methods were also developed for urine, faeces and tissues *(17,25)*. The only analytical method for quantification in soil used fluorescence detection *(26)*. To this end, no method is available for quantification of zearalenone in

sewage sludge and manure. HPLC-MS/MS is the state of the art technique for organic compound analysis, such as zearalenone. In particular for solid matrices, the crucial step in HPLC-MS/MS is the ionisation of the analyte, which is affected by co-extracted matrix compounds *(27-29)*. So far, the only effective way to achieve precise and accurate results in the presence of matrix compounds is the use of isotope labelled IS, in our case six fold deuterated zearalenone (D_6-zearalenone). One important objective of this work is to demonstrate that the use of deuterated IS is an effective way to overcome matrix related problems and compensating sample losses during the analytical procedure. In the past, D_6-zearalenone was used in aqueous matrices *(29,30)* and D_2-zearalenone in cereal products *(24)* for zearalenone quantification.

In this study we present for the first time a series of accurate and precise analytical methods for several environmentally relevant types of samples. To our knowledge this is the first time D_6-zearalenone is used as IS for quantification of zearalenone in various solid matrices. The analytical methods are optimized and validated for corn flour, soil and sewage sludge, and validated for corn straw, wheat flour, wheat straw and manure. Their application is demonstrated in real soil and plant samples from a local field study and in manure and sewage sludge samples from monitoring studies throughout Switzerland. Due to the similar analytical behaviour, several metabolites of zearalenone were also monitored in all real samples using the procedure as described in Hartmann et al. *(29)* for various aqueous samples.

3.2 Method and materials

3.2.1 Chemicals

Zearalenone [CAS-No. 17924-92-4] (≥99%), was purchased from Sigma (Buchs, Switzerland). D_6-zearalenone was prepared in our own laboratory by base-catalyzed hydrogen-deuterium exchange as described in Miles et al. (31). Hydrogen–deuterium exchange takes place at the positions C-3, C-5, C-5’ and C-7’. The purity of D_6-zearalenone was tested by injecting a pure IS solution, scanning for D_0- to D_6-zearalenone. The relative zearalenone amount was less than 0.1%. Ratios of the different grades of deuterated zearalenone did not change over time. Therefore, D_6-zearalenone was found to be a suitable IS and the other deuterated zearalenone products did not influence quantification *(29)*. Methanol, toluene, cyclohexane,

acetone and acetonitrile (all HPLC-grade) were purchased from Scharlau (Barcelona, Spain). Water was purified by a Milli-Q system from Millipore (Volketswil, Switzerland). N_2 (99.99995%) was purchased from PanGas (Dagmersellen, Switzerland). A zearalenone stock solution holding concentrations of 500 mg/L was prepared in pure methanol. The IS solution was prepared in methanol and had a concentration of 2 µg/mL. IS solutions contained deuterated resorcyclic acid lactone analogues *(29)* for analysis of real samples, but only D_6-zearalenone for method development. Aqueous calibration standards holding zearalenone equivalent to the concentration range of 0.5-100 ng/L and 100 ng/L of the IS were prepared in Milli-Q water from the methanol stock solution each time a new batch of samples was analysed. Stock solutions and dilutions thereof were stored in the dark at -20 ℃ and 4 ℃, respectively.

3.2.2 Sample collection and preparation

Plant material: Wheat- and corn plant materials were collected manually from a 0.2 hectare field site at Zürich-Reckenholz, Switzerland. For method development and validation, 500 wheat and 50 corn plants were selected at harvest. Kernels of wheat and corn were separated from the rest of the plant, in the following referred to as straw. This material was naturally contaminated with zearalenone and additional spiking thus not necessary. Real samples were collected several times before, and at the time of harvest as follows: 500 wheat plants and 50 corn plants were taken from randomly selected locations over the field and divided into their organs. Wheat plants were divided into kernels, spelts, stalks, stalks of the ears and leaves. Similarly, corn plants were divided into kernels, stalks, spindles, leaves and leaves of the spindles. Samples were stored at -20 ℃. Before extraction, the samples were dried at 40 ℃ until weight constancy was achieved, but for at least 48 h, ground and sieved to 0.5 mm using a ZM1 centrifuge mill (Retsch GmbH, Germany). Real samples were analysed within 48 h after drying.

Soil: Soil samples were taken with a split tube core sampler (2.5 cm diameter). Fifteen to 20 samples were taken from randomly selected locations over the field site at Reckenholz and pooled to one composite sample. Topsoil (0-5 cm) samples were used for method development and validation. As these samples were naturally contaminated with zearalenone, additional spiking was not necessary. Real samples

were taken in depths of 0-10 cm, 10-20 cm and 20-40 cm. Further sample handling was as stated previously for the plant materials.

Manure: For method development and validation, 100 L of manure (swine:cattle; 1/1) were obtained from our research station in Tänikon (Switzerland) and then spiked with zearalenone to a resulting concentration of around 100 ng/L. To account for real situations, the spiked manure aged for 96 h before further treatment. Real samples were taken from selected farms within the Swiss soil monitoring network (NABO). Due to the same origin and its similar composition, liquid manure (urine and feces) and dung (feces and straw material) were handled in the same way. In this study the term manure stands for both liquid manure and dung. Before sampling, liquid manure containers at the selected farms were homogenized for two h using the stirring unit. Real samples were taken with a PVC-pipe (5 m length, 7 cm diameter) for vertical sampling. Five samples were taken from different depths and filled in a 30 L bucket to form a composite sample. One litre of this composite sample was taken for analysis. This sampling device for liquid manure was applied in previous studies in Switzerland *(32)*. Dung samples were taken manually. Ten samples were taken from randomly selected spots from the dung pile and pooled to one composite sample of 1 kg. The whole composite sample was used for analysis. Samples were handled as described above for plant materials, except that they were dried at 105 ℃ for at least 96 h.

Digested sewage sludge: For method development and validation, 100 L sewage sludge were taken from the WWTP Werdhölzli (Zürich, Switzerland) and then spiked with zearalenone to a resulting concentration 100 ng/L. The spiked sludge aged for 96 h before further treatment. Real samples were taken from 30 WWTP throughout the midlands of Switzerland. Selection criteria were: the WWTP had to be part of the existing monitoring networks such as SEA (Observation of the Metabolism of the Anthroposphere) *(33)*; and, possible zearalenone sources such as agricultural land, animal and human excretion, and wastewater from feed and food industry had to be covered. Samples were taken at four different times from May 2006 to February 2007. Before sampling, the sludge holding tank at the selected WWTP was mixed using the stirring unit getting a homogenous sludge distribution. Real samples were taken manually *(33)* using a one litre Niskin-bottle. Three individual samples were then pooled to one composite sample. Further sample handling was as described above for manure.

3.2.3 Extraction method

Plant material: In contrast to all other investigated matrices, a certified reference material was available for corn flour (zearalenone in corn flour: 60 ± 9 ng/$g_{dry\ weight\ (dw)}$; biopure, Tullin, Austria). We therefore knew what zearalenone content we needed to achieve and whether extraction was complete or not. The widely used solvent composition for corn flour extraction (acetonitrile/Milli-Q water (84:16, v/v)) *(34-36)* yielded to complete extraction after 2 h. This mixture is also frequently applied for wheat samples *(37)*, therefore this procedure was not further optimized, but selected for all investigated corn and wheat materials. Liquid solvent extraction of 1 g plant material with 50 mL solvent was carried out on a SM-30 orbital shaker (Edmund Bühler GmbH, Hechingen, Germany).

Soil: Three different extraction methods were compared, i.e., liquid solvent, Soxhlet, and accelerated solvent extraction (ASE, ASE200, Dionex Corporation, Sunnyvale, Ca). ASE and Soxhlet extraction exhibited similar extraction rates and were superior to liquid solvent extraction. Due to the easier handling, Soxhlet extraction was preferred over ASE. Before extraction, soil samples were homogenized with a turbula (Turbula System Schatz, Willy A. Bachofen AG, Muttenz, Switzerland) for 15 min. Five grams of the sample were extracted with 150 mL solvent in a 100 mL Soxhlet extractor. The optimization of extraction parameters was performed with aged soil gathered from the field site as described above. Three replicates were extracted for every tested solvent, solvent mixture and extraction duration. Different solvents (Milli-Q water, acetonitrile/Milli-Q water (84:16, v/v), acetonitrile, methanol, methanol/toluene (80:20, v/v), toluene, acetone/toluene (80:20, v/v), acetone, acetone/cyclohexane (80:20, v/v), cyclohexane), ranging from non-polar to polar ones were tested at 6 h extraction time. Extraction duration (6, 18, 36 h) dependence was eventually tested for the most effective solvent.

Manure: Liquid solvent extraction was selected for manure, because ASE and Soxhlet extraction both led to very dirty extracts which made further concentration steps almost impossible due to oily residues. Since an important component of manure is plant material, it was extracted with the same solvent mixture as used for plant material and no solvent optimization was conducted. Extraction duration was tested at 2, 4 and 120 h. Two grams of manure were extracted with 50 mL solvent mixture.

Digested sewage sludge: Sludge samples were extracted with Soxhlet. Liquid solvent extraction was much less effective and ASE led to similar difficulties as described above for manure. Before extraction, sludge samples were homogenized with the turbula for 15 min. Five grams of the sample were extracted with 150 mL solvent. The optimization of extraction parameters was performed with spiked and aged sludge prepared as described above. Optimization of extraction was performed as described above for soil.

3.2.4 Extract processing

Plant material: The IS (50 µL, 2 ng/µL) was spiked to a 1 mL aliquot of the total extract. This subsample was transferred to a 10 mL microreaction vial (Supelco, Bellfonte PA, USA) and then evaporated to dryness with a gentle stream of nitrogen at 50 °C for approximately 10 min. The dried extrac t was reconstituted with 1 mL of acetone. For further clean up, the extract was centrifuged (Labofuge 200, Heraeus Sepatech GmbH, Osterode, Germany) at 4000 rpm for 5 min. The supernatant was transferred to a microreaction vial, evaporated to dryness again, and reconstituted with 300 µL of Milli-Q water/acetone (50:50, v/v). The percentage of acetone for reconstitution was tested between 20% and 80% with regard to chromatographic separation, peak shape and resolution of the analytes. At 50% acetone they were fully reconstituted and neither separation nor peak shape were negatively influenced. The extracts were stored in the dark at 4 °C and an alyzed *(29)* within 24 h. Prior to measurement, the extract was filtered with a syringe filter (13 mm Syringe Filter, 0.2 µm PTFE, BGB Analytik AG, Böckten, Switzerland) and transferred to a 350 µL amber glass vial.

Soil: The IS (50 µL, 2 ng/µL) was added to the total soil extract. The total extract was evaporated to 1 mL in a 12 fold parallel evaporator (Syncore Analyst, Büchi Labortechnik AG, Flawil, Switzerland), transferred to a 10 mL microreaction vial and evaporated to dryness using a gentle stream of nitrogen at 50 °C for approx. 10 min. Reconstitution and further extract treatment were as described above for plant material, except that the final filtration was only performed in the case of extract coagulation in the stored glass vial.

Manure: The IS (50 µL, 2 ng/µL) was spiked to a 5 mL aliquot of the total extract. This subsample was evaporated to dryness with a gentle stream of nitrogen at 50 °C

for approximately 30 min. The dried extract was reconstituted with 1 mL of acetone and further treated as described above for plant material.

Digested sewage sludge: The IS (250 µL, 2 ng/µL) was added to the total sludge extract. The total extract was evaporated to 1 mL with the Syncore system, transferred to a 10 mL microreaction vials and evaporated to dryness using a gentle stream of nitrogen at 50 ℃ for approx. 10 min. The dried extract was reconstituted with 1 mL of acetone and further treated as described above for plant materials.

3.2.5 Chromatographic separation and mass spectrometric detection

LC-MS/MS was performed on a Varian 1200L LC-MS instrument (VarianInc., Walnut Creek, CA). The resorcyclic acid lactones were separated on a 150 x 2.0 mm, 3 µm Polaris Amide-C18 column (VarianInc., Walnut Creek, CA) at room temperature by applying the following elution gradient: 0 min 0% B (100% A), 3min 0% B, 4 min 40% B, 25.5 min 67.5% B, 26 min 100% B, 29 min 100% B, 30 min 0% B, 35 min 0% B; with eluent A consisting of Milli-Q water/ACN (95/5, v/v) and eluent B of Milli-Q water/ACN (5/95, v/v). Both eluents were buffered with 10 mM ammonium acetate (pH=6.8). The injection volume was 50 µL and the mobile phase flow rate was 0.2 mL/min. Interface parameters of the LC-MS/MS were as follows: needle voltage -2000 V, nebulizing gas (compressed air) 4.21 bar, capillary voltage -54 V, drying gas (N_2, 99.5%) 300 ºC and 1.59 bar, shield voltage -600 V. Detection of the resorcyclic acid lactones was performed in the (-)ESI mode. The collision cell gas (Ar, 99.999%) pressure was 2.0 e^{-6} Torr and the detector voltage was set to 2000 V. Detailed information about retention times, precursor and product ions are given in Hartmann et al. *(29)*. Although the analytical methods were optimized and validated for zearalenone only, the other resorcyclic acid lactones were monitored as well in all investigated samples.

3.2.6 Method validation parameters

Ion suppression was evaluated by comparing the analyte signals obtained from injection of the same amount of analyte dissolved in the final extract from the various matrices, and in the respective pure solvent. Standard addition to the final extracts was carried out to yield concentrations equivalent to 5, 10, 25, 50 and 100 ng/mL. Curves were obtained by plotting measured analyte peak areas against

corresponding analyte concentration levels in pure solvent and in extracted matrix, respectively. Linear regression was performed for each curve. The ion suppression (expressed in %) was quantified as 1 minus the ratio between the slope of the curve obtained for the extracted matrix and the slope of the curve for the pure solvent.
Absolute recoveries over extraction, cleanup and quantification were determined for zearalenone in all described matrices. They were spiked prior to extraction with 500 ng (plant material), 5 ng (soil), 100 ng (manure) and 25 ng (sewage sludge) zearalenone per g_{dw}. In the case of corn flour, absolute recovery was obtained by the extraction of the reference material mentioned above. The different spike levels were selected to match with the expected native zearalenone concentration in each matrix. Five replicates were prepared for every matrix. The extraction was started 24 h after the spiking with zearalenone. IS was added prior to the analysis by HPLC-MS/MS. The absolute method recovery was defined as the ratio between the quantified and the spiked amount. Native amounts as determined in respective blank samples were accounted for. Relative recoveries over cleanup and quantification were obtained again for all matrices. Zearalenone spike levels and replicates were the same as described above for the absolute recoveries but the IS and zearalenone were spiked right after the extraction step in the extract or the aliquot thereof. The relative recoveries were defined as the ratio of the quantified and the spiked amount. The precision of the analytical method was defined as the mean relative standard deviation of these five replicates at the chosen concentration levels. For all matrices, the method quantification limit was calculated based on a signal to noise ratio of ten (S/N=10) of spiked samples in the case of soil and sludge and of blank samples in the case of manure and plant material.

3.3 Results and discussion

3.3.1 Extraction

Plant materials and manure: Because of the good agreement between the quantified and certified concentration of the corn flour reference material (see below) with a commonly used method (2 h extraction with acetonitrile/Milli-Q water 84:16, v/v), further solvent and extraction time optimization for plant material was not performed. Due to the similarities of plant material and manure (see above), the same solvent was chosen for the latter and only extraction time was optimized. The

normalized extraction efficiency was 100 ± 15%, 97 ± 7% and 87 ± 6% at 2, 4 and 120 h, respectively. Due to the very similar extraction efficiency at 2 and 4 h but lower relative standard deviation at 4 h, method validation and real sample extraction were performed with acetonitrile/Milli-Q water (84:16, v/v) during 4 h by liquid solvent extraction.

Soil: Figure 3.1A shows the Soxhlet zearalenone extraction efficiency for several solvents and mixtures thereof. All numbers were normalized to the highest quantified concentration, which was 6.4 ng/g_{dw}. Non-polar solvents such as toluene and cyclohexane were clearly inferior to polar solvents such as acetone, acetonitrile, methanol or mixtures with 80% of polar solvents. Maximum extractability was achieved with pure methanol. There was a clear difference in extraction efficiency between Soxhlet and simple liquid solvent extraction with acetonitrile/Milli-Q water (84:16, v/v) (data not shown here), probably due to higher solvent temperature and repeated extraction cycles with fresh solvent during Soxhlet extraction. The optimization of the extraction time was performed with pure methanol. The normalized extraction efficiency was 82 ± 4%, 100 ± 18% and 84 ± 18% at 6, 18 and 36 h, respectively. Consequently, the final Soxhlet extraction method chosen for method validation and real sample extraction was performed with methanol for 18 h.

Digested sewage sludge: The solvents yielding best zearalenone extractabilities for soil, i.e. methanol and methanol/toluene (80:20, v/v), were most efficient for sewage sludge as well (Figure 3.1B). Again, polar solvents performed somewhat better than non-polar ones. The optimization of the extraction time was performed with methanol/toluene (80:20, v/v). The standardized extraction efficiency was 94 ± 7%, 100 ± 1% and 89 ± 7% at 6, 18 and 36 h, respectively. The final method chosen for method validation and real sample analysis was Soxhlet extraction with methanol/toluene (80:20, v/v) for 18 h.

3.3.2 Method validation parameters

Ion suppression of zearalenone occurred in all investigated matrices but to a very different extent. Numbers ranged from 8 to 54% for plant materials and from 8 to 74% for environmental matrices (Table 3.1). For wheat flour ion suppression was very similar to numbers reported by Zöllner et al. *(38)* whereas the level for corn flour was clearly lower than the reported 70% by Berthiller et al. *(23)*. Interestingly, ion

suppression in straw was considerable higher than in flour for both wheat and corn. Ion suppression levels for manure were considerably higher than reported by Songsermsakul et al. *(17)* for urine and faeces of horses.

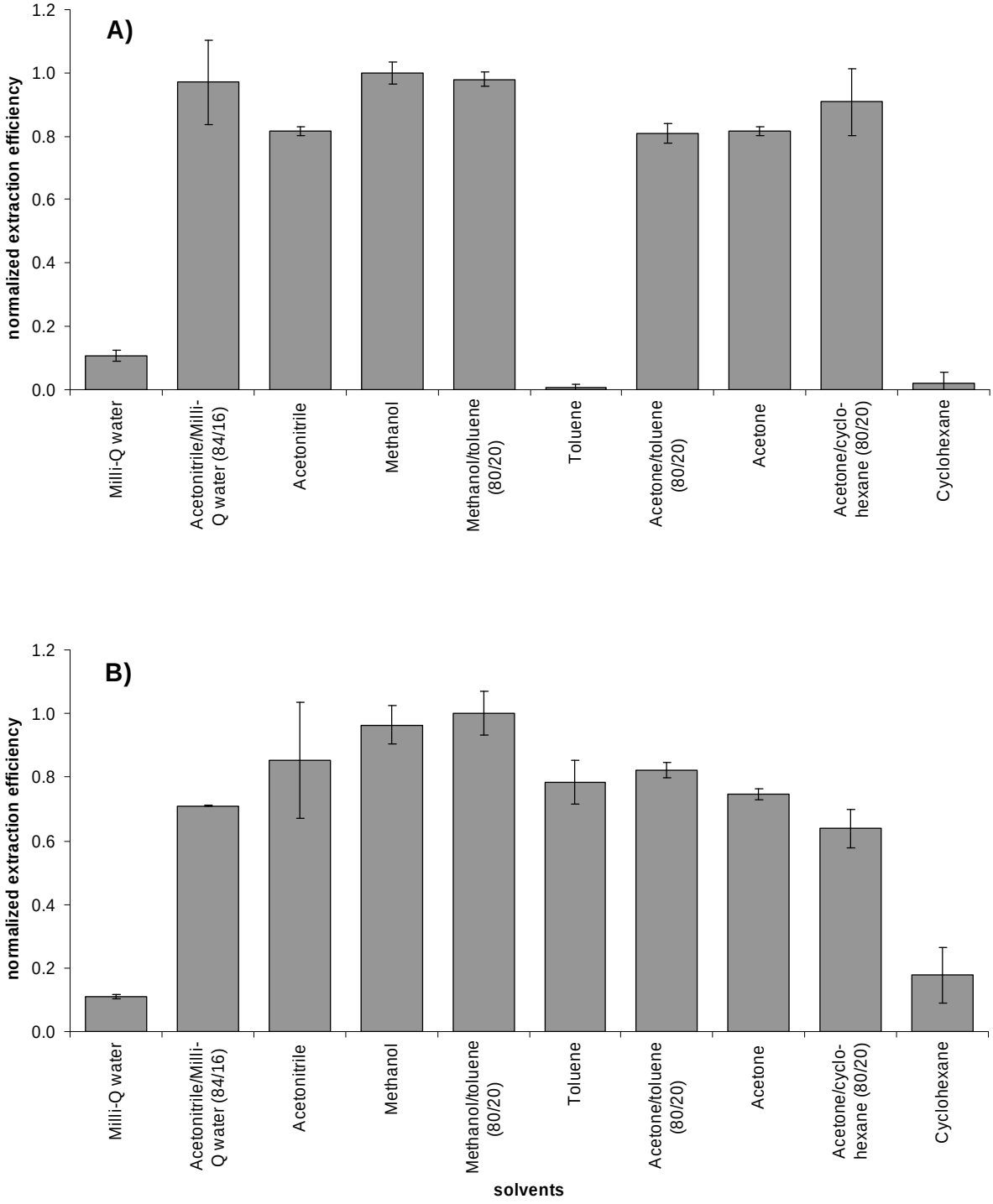

Figure 3.1: Normalized Soxhlet extraction efficiency from soil (A) and digested sewage sludge (B). Error bars represent standard deviations of three individual extractions.

Absolute method recoveries were determined in all matrices, but at different

concentrations as described above. The lowest number (30%) was achieved for sewage sludge (Table 3.1). For all other investigated matrices absolute recoveries were satisfactory with numbers above 70%. The absolute recovery obtained for soil (105%) was very similar to the one reported by Mortensen et al. *(26)*. For manure, the absolute recovery of 76% was somewhat lower than reported for horse feces and urine *(17)*. In sewage sludge, about 60% of the analyte remained in the discarded coagulated oily and solid extract fraction (see above), as evidenced by the correspondingly lower IS signal when spiked after extraction as opposed to before analysis. This also indicates that extraction was almost complete and losses of analyte primarily occurred during the following steps. Because the IS was spiked right after extraction for quantification of real samples, the described analyte losses were compensated by respective losses of the IS. However, the method quantification limit rose due to less analyte per injection. The absolute recovery of 104% for corn flour deserves particular attention: as this number was obtained from a certified corn flour with known zearalenone content, no further optimization of the extraction method was necessary.

Table 3.1) Method validation parameters for investigated agro-environmental matrices [a]

Matrix	Ion suppression [b]	Absolute recovery	Relative recovery	Method precision [c]	Method quantification limit [d]
	[%]	[%]	[%]	[%]	[ng/g_{dw}]
Wheat flour	8	70 (9.1) [e]	107 (10.9) [e]	3	3.3
Wheat straw	35	82 (1.7) [e]	97 (5.1) [e]	2	12.0
Corn flour	15	104 (4.8) [f]	110 (17.1) [e]	9	3.2
Corn straw	54	97 (13.5) [e]	106 (9.5) [e]	14	26.2
Soil	8	105 (3.8) [g]	106 (4.4) [g]	9	0.7
Manure	74	76 (3.5) [h]	86 (8.2) [h]	7	12.3
Sewage sludge	49	30 (21.3) [i]	113 (1.7) [i]	7	6.8

[a] Numbers in brackets show standard deviation of five replicates.
[b] Between 5 and 100 ng/mL extract
[c] Relative standard deviation of five replicates of naturally contaminated (plant material and soil) and aged (manure and sewage sludge) samples
[d] S/N=10
[e] Spiked with 1000 ng/g
[f] Reference material containing 60 ng/g
[g] Spiked with 5 ng/g
[h] Spiked with 500 ng/g
[i] Spiked with 25 ng/g

Relative recoveries were established in all matrices at the same concentrations as for absolute recovery determination, except for corn flour. They ranged from 86% for manure to 113% for sewage sludge (Table 3.1). These numbers show very well that

the IS behaved almost the same as zearalenone during all analytical steps after extraction, independent on the matrix. Therefore analyte losses during extract processing such as in the case of sewage sludge (see above) and ion suppression were compensated by similar losses of D_6-zearalenone. This fact renders the analytical methods very robust to matrix variation, and is in accordance with earlier results with different types of aqueous samples *(29)*.

The precision ranged from 1.8% to 13.5% (Table 3.1). These numbers lie well within acceptable values for solid matrices *(39)* and commonly reported ranges for plant materials *(22,40)* and soil *(26)*. The precision for manure is comparable to those reported for horse feces and urine *(17)*.

Method quantification limits were between 0.7 ng/g_{dw} for soil and 26.2 ng/g_{dw} for corn straw and generally correlated with ion suppression (Table 3.1). These levels are higher than reported for plant materials *(23,40)* and horse feces *(17)* due to less specific clean up steps. However, obtained method quantification limits were sufficiently low for our applications.

Note again that the extraction step was optimized only for corn flour, soil and sewage sludge, whereas a common extraction solvent (acetonitrile/Milli-Q water (84:16, v/v)) was selected for other plant materials and manure. The satisfactory analytical figures of merit for all matrices (Table 3.1) justify this approach and indicate that our assumptions about similarities (see above) of the other plant materials and manure were appropriate.

3.3.3 Application to real samples

We are currently using the analytical methods presented here to study the zearalenone input and distribution on *Fusarium*-infected wheat and corn fields at Reckenholz *(29,30,41)*. Additionally, manure samples were investigated from the Swiss soil monitoring network (NABO) and sewage sludge samples were analyzed from our own monitoring network throughout Switzerland. Table 3.2 shows a compilation of the measured zearalenone concentrations in these matrices, which are discussed in more detail as follows. A further evaluation of the environmental distribution and relevance of zearalenone is carried out in Hartmann et al. *(20)*.

Plant materials: Depending on the plant organ, zearalenone concentrations (Table 3.2) in wheat samples ranged from 86 ng/g_{dw} to 16.7 µg/g_{dw}. Similar concentrations of

126 ng/g_{dw} to 13.8 µg/g_{dw} were quantified in corn. These ranges fit well with data found in literature *(42)*, although plant organs are usually not distinguished or specified. Our data show that zearalenone concentrations vary much within the different plant organs. Concentrations in some plant organs such as wheat spelts or corn spindles were significantly higher than in the kernels, which are removed by harvest. This indicates that, depending on the agricultural practice, a substantial zearalenone fraction remains on the field after wheat and corn harvest and is thus still potentially available to the environment.

Table 3.2) Zearalenone concentrations quantified in the investigated agro-environmental matrices

Matrix	n	Concentrations		
		Min [ng/g_{dw}]	Median [ng/g_{dw}]	Max [ng/g_{dw}]
Plant material [a]				
Wheat kernels (flour)	3	260	2228	2565
Wheat organs [b] (straw)	10	86	1378	16653
Corn kernels (flour)	4	270	368	399
Corn organs [c] (straw)	9	126	1286	13767
Soil	80	n.d.	0.4	8
Manure				
From swine	5	17	160	333
From cattle	6	24	90	197
Mixed	7	8	40	118
Cattle dung	12	21	41	70
Sludge	85	n.d.	n.d.	37

[a] Collected at the time of harvest
[b] Investigated wheat organs were: leaves, glumes, stalks and stalks of the ears
[c] Investigated corn organs were: leaves of the stalks, leaves of the spindles, spindles and stalks
n: Number of samples
n.d.: Not detected

Soil samples: Zearalenone concentrations in the soil (Table 3.2) ranged from not detectable up to 7.5 ng/g_{dw}. Highest concentrations were quantified after a rain event at a time when the wheat plants were heavily infected by *Fusarium* fungi, and in topsoil samples. For instance, in November 2005 zearalenone concentrations were 3.8, 1.2 ng/g_{dw} and below method quantification limit, but above the method detection limit, at depths of 0-10 cm, 10-20 cm and 20-40 cm, respectively. A decreasing zearalenone content with increasing sampling depth seems plausible and gives further credibility to the presented analytical methods, including sampling and sample preparation procedures.

Manure samples: All investigated manure samples contained zearalenone, however at very different levels. Table 3.2 compiles the zearalenone concentrations for different types of manure. Average zearalenone concentrations were between 50 and 150 ng/g_{dw}, or between 2 and 8 ng/$g_{wet\ weight}$. From the daily manure production of cattle (55 L) and swine (4.4 L), an average zearalenone excretion per day can be calculated. These levels were between 25 and 350 µg/day/animal for swine and cattle, respectively. Based on the yearly amounts of manure applications and the corresponding areas (data obtained from the respective farmers), these amounts translate in an average zearalenone load of about 50 to 150 mg zearalenone per hectare and year. In four out of the 30 investigated samples, α-zearalenol and/or β-zearalenol were quantified at levels between 12 and 179 ng/g_{dw}. The other 26 samples did not contain any detectable amounts of these metabolites, which are known to be excreted by farm animals *(43)*. Corresponding with the literature *(14)*, only α-zearalenol was found in swine manure, whereas β-zearalenol dominated in cattle manure.

Digested sewage sludge samples: In total, 85 digested sewage sludge samples were analyzed from 30 WWTP between May 2006 and February 2007. In 24 out of these samples zearalenone was detected, but in only two cases the levels were high enough for quantification. These two zearalenone concentrations were 36.9 and 12.5 ng/g_{dw}. The respective WWTP were located at Wenslingen (Canton of Basel-Land) and Märstetten (Canton Thurgau), and received wastewater via combined sewer systems from private households and surface runoff. They had the highest winter wheat area to inhabitants ratio, which indicates that *Fusarium* infested wheat fields were possibly responsible for this zearalenone occurrence. Using the equation $c_w = c_s / (f_{oc} * K_{oc})$, hypothetical zearalenone concentrations in the corresponding waste water effluent can be calculated. Including the f_{oc} from the two sewage sludge samples (0.225 and 0.229, respectively) and a K_{oc} of 4250 L/kg (own soil sorption experiments), hypothetical zearalenone concentrations in the wastewater effluents resulted in 34 and 13 ng/L. These numbers correspond well with data reported in the literature *(18)*.

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Chapter 4: Fate of zearalenone on wheat and maize fields

Occurrence of Zearalenone on *Fusarium Graminearum* Infected Wheat and Maize Fields in Crop Organs, Soil and Drainage Water

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Abstract

The mycotoxin zearalenone (ZON) is a very potent natural endocrine disrupting chemical, produced by *Fusarium graminearum* fungi growing on crops such as wheat and maize. Although it is well investigated in food and feed, very little is known about its environmental fate and behavior. Here, we report on the occurrence of ZON on *Fusarium graminearum* infected wheat and maize fields in crop organs and soil, and its emission via drainage water. ZON amounts in the investigated crops and topsoil were between 6.1 and 25.0, and up to 5.6 g/ha, respectively. ZON concentrations in drainage water were in the low ng/L range with a maximum of 35 ng/L. Cumulated ZON amounts emitted via drainage water ranged from 0.1 to 4.3 mg/ha, depending on the crop cultivated in the respective period. This corresponds to fractions between 0.001 and 0.070% of the initially present ZON amount in the plants. Due to the low concentrations emitted via drainage water, it can be assumed that ZON contributes little if at all to the overall estrogenicity of major surface water bodies. However in small creeks, mainly fed by agricultural runoff, ZON might be present in environmentally critical concentrations at times of *Fusarium graminearum* infections.

4.1 Introduction

Zearalenone (ZON) is known as a very potent endocrine disrupting chemical. Its relative estrogenic potency *(1)* and binding affinity to the estrogen receptor *(2)* are close to that of 17β-estradiol, and orders of magnitudes higher than for notorious synthetic endocrine disruptors such as bisphenol A, DDT or atrazine *(1)*. ZON is the most prominent representative of the resorcyclic acid lactones (RALs) (see supporting information SI in appendix, Figure S1). It is produced by *Fusarium* fungi, in particular by *Fusarium graminearum* growing on maize, wheat and other cereals *(3)*, which cause *Fusarium* head blight on wheat and ear/stalk rot on maize. Because both maize and wheat crops can be infected by *Fusarium graminearum*, the crop rotation wheat after maize is problematic due to fungi survival on maize plant debris remaining after harvest on the field, from which infestation of the following wheat takes place. The extent of *Fusarium graminearum* infection on wheat and maize, and subsequent ZON contamination is determined by several factors: 1) climatic conditions, 2) crop rotation, 3) soil cultivation and 4) susceptibility of crop varieties *(4)*. In recent years the problem has worsened probably due to the alteration of the cropping system, reduced soil tillage, and, possibly, changing climatic conditions *(4)*.

Plenty of effort has been spent to investigate the occurrence and fate of ZON in food and feed products, and its estrogenic effects particularly in domestic animals. ZON was reported to occur regularly in agricultural food and feed products around the world *(5,6)*. An overview in Switzerland *(5)* showed that 13% of the investigated samples contained ZON, mostly in the low µg/kg range. *Fusarium graminearum* infested feeding or litter can cause severe reproductive and infertility problems in husbandry animals *(7,8)*, in particular for swine.

In contrast to the extensive investigations in the agricultural and food context, very little is known about the environmental distribution of ZON. Several publications reported the occurrence of ZON in surface waters *(9,10)*, and in- and effluents of waster water treatment plants *(9,11,12)*. Concentrations ranged from below detection limit up to 60 ng/L for individual samples. In some cases also other RALs such as α-zearalenol (α-ZOL), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) were detected at similar levels as ZON (10,11,13). Unfortunately in most of these publications possible sources of the RALs were not elucidated. In one case the presence of RALs was associated with cattle excretion of animals fed with growth promoters *(9)*. However,

the environmental distribution of ZON and its emission from *Fusarium graminearum* infected wheat and maize fields into the aqueous and non-aqueous environment have not yet been investigated.

All six RALs were investigated in this field study. Table 4.1 compiles available physio-chemical data for ZON, α-ZOL and for 17β-estradiol. This comparison is of interest, since their aqueous solubilities and octanol-water partition constants (K_{ow}) are in the same order of magnitude *(14)*, and because the estrogenicity of endocrine disruptors is often related to these of natural steroid hormones. Test results of different cereal washing procedures revealed that up to 61% of ZON desorbed from contaminated maize kernels into distilled water *(15)*. This indicates some environmental aqueous phase mobility. Simultaneously, with an estimated $\log K_{ow}$ of 3.58 and calculated $\log K_{oc}$ of 3.89 (Table 4.1), the compound very likely exhibits a certain potential for sorption and retention in soil systems, depending among other potential sorbents on the organic carbon fraction. Its abiotic stability during milling, food processing, heating, etc. is considerable *(16)* and it must for this reason be assumed that ZON could be rather chemically persistent in the environment as well. Data on biotic transformation of ZON generally indicate as a main metabolization pathway the reduction of the 6'-keton to yield α- and β-ZOL *(17)*. Mortensen et al. *(18)* described the fate of ZON in soils and reported an overall dissipation half lives of 6 to 11 days, including degradation and irreversible sorption, but without quantifying degradation products.

Table 4.1) Physio-chemical properties for ZON, α-ZOL and 17β–estradiol

Analyte	Aqueous solubility	Log K_{ow}	Log K_{oc}	pK_a	Half lives
	[mg/L]				[day]
ZON	5 [a]	3.6 [b]	3.9 [c]	7.6 [d]	6–11 [e]
α-ZOL [f]	4–28 [g]	3.1–3.5 [g]	n.a.	8.4, 11.4 [h]	7–30
17β–estradiol	13 [i]	4.0 [k]	3.1-3.7 [l]	10.4 [m]	3 [n]

[a] Ref. *(29)*; [b] Estimated *(30)*; [c] Calculated by EPIwin v3.1 *(18)*; [d] Estimated *(31)*; [e] Dissipation in soil *(18)*; [f] Ref. *(24)*; [g] At pH=5, 7, 9; [h] pK_{a1} and pK_{a2}; [i] Ref. *(14)*; [k] Calculated by HyperChem Software *(14)*; [l] Ref. *(32)*; [m] Estimated *(14)*; [n] Aerobic *(28).*

The aim was to investigate the environmental fate of RALs at such fields and to quantify the amounts emitted into surface waters via drainage water discharge. To the best of our knowledge, this is the first time that the environmental exposure to estrogenic mycotoxins is examined in such detail.

4.2 Experimental section

4.2.4 Field site description and instrumentation

The study site (47° 25’ 45’’ N / 8° 30’ 53’’ E) was located nearby the agricultural research station Reckenholz, north of Zürich, Switzerland. It was divided into two 0.2 hectare plots with a gently slope of 1-2°. The tops oil was classified as a medium to heavy textured gleyic cambisol with 31% clay, 30% sand, 39% silt and an organic carbon fraction of 2%. Further soil parameters are given in the supporting information (Table S1). Both plots were individually drained by two long and two short drainage tubes, which met respective main drainage tubes (Figure 4.1) with a diameter of 15 cm. Drainage tubes were located in a depth of 80 to 90 cm. The groundwater table was recorded at 30 minute intervals during the first year of the study with three piezometers located on a transect (Figure 4.1) across the two plots. Its depth ranged from 100 to 125 cm (Figure S2 B) and thus was permanently below the drainage system. Both main tubes ended in sampling ducts, which were equipped with flow meters and automated samplers (7612 ISCO with a 730 bubbler module, both from Teledyne Isco Inc. Lincoln, USA) for discharge proportional drainage water sampling. Precipitation data were gathered in intervals of ten minutes (SI, Figure S2 A) by the meteorological station (Reckenholz, 443 meter above sea level, 47°25’ 40’’ N / 8°31’ 04’’ E, Meteoschweiz) *(19)* 300 m nearby the field site.

4.2.2 Field cultivation and Fusarium graminearum infestation

The goal of the cultivation practice was to achieve a worst case scenario in terms of maximum *Fusarium graminearum* infestation of the crops. Therefore, several actions favorable *(4)* for *Fusarium graminearum* infestation were taken: a) the common crop rotation winter wheat after maize was chosen, b) wheat (Levis) and maize (Birko) varieties, susceptible to *Fusarium graminearum* infestation where selected, c) to create optimal conditions for *Fusarium graminearum* to infect the wheat plants, no till soil cultivation was performed. Thereby, the maize debris were not covered by earth as it is the case when the plough is used. Moreover maize debris were not chaffed to slow down the decomposition of the plant material or to assure the overwintering of the fungus and the production of abundant infectious spore material in spring and especially during the flowering time of the wheat, and d) the winter wheat fields were artificially infected during flowering with a spore suspension containing a mixture of

three *Fusarium graminearum* strains (CBS 121291, CBS 121292, CBS 121293). The two plots were both cultivated as described above, but crop rotation started at different times. Crop rotation at the upper plot (Figure 4.1, A) was as follows: 1) winter wheat from November 2004 to July 2005, 2) oil radish as intercrop from July 2005 to May 2006, 3) maize from May 2006 to November 2006 and 4) winter wheat from November 2006 to July 2007. Crop rotation at the lower plot (Figure 4.1, B) was as follows: 1) winter wheat from November 2004 to May 2005 (no artificial *Fusarium graminearum* infection and premature harvest), 2) maize from May 2005 to November 2005, 3) winter wheat from November 2005 to July 2006 and 4) grassland from July 2006 to July 2007.

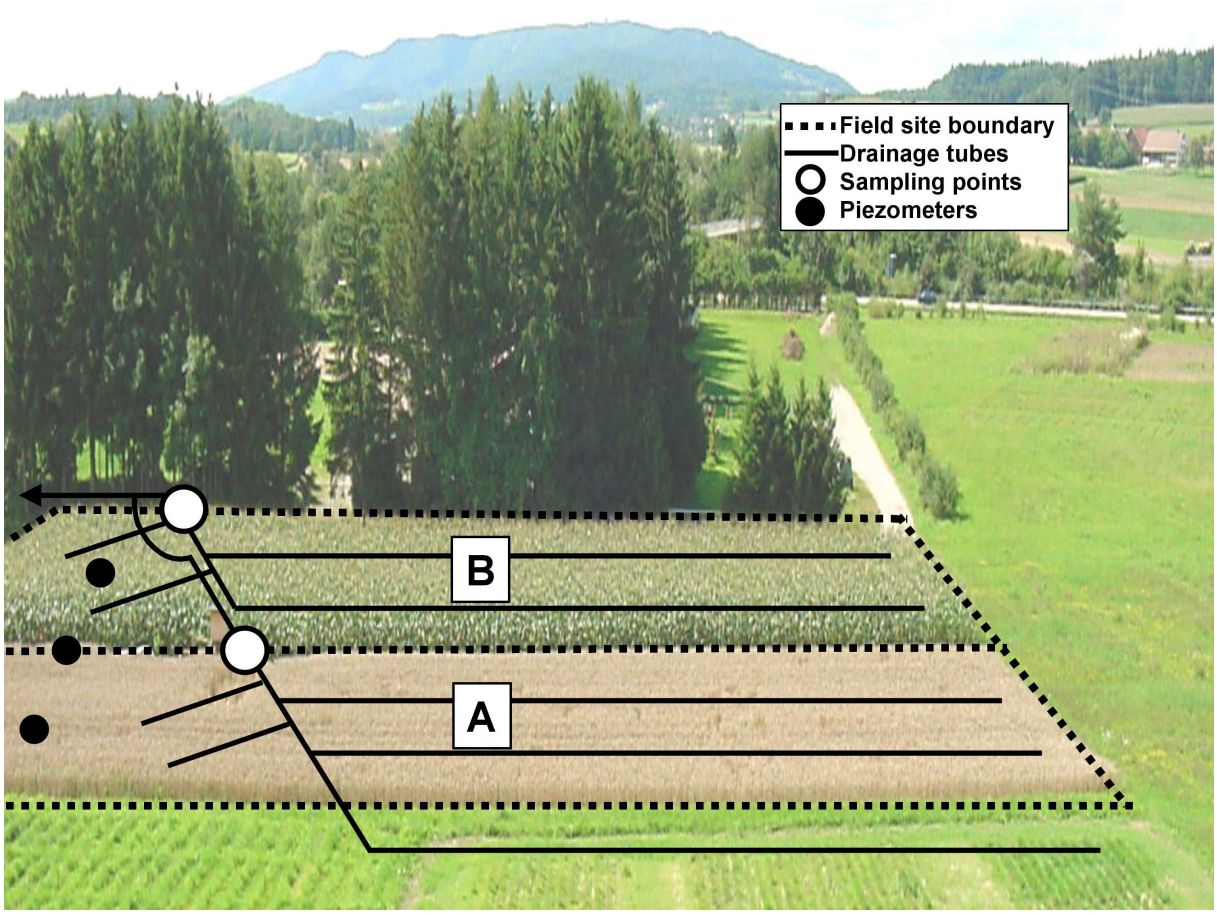

Figure 4.1: Field site at the research station in Zurich-Reckenholz, divided in the two plots A) and B). Dashed lines: field site boundaries, solid lines: drainage tubes, circles: sampling ducts, black dots: piezometers. For further details, see text and SI.

4.2.3 Sampling and analytical procedures

To assess the distribution of the RALs, crop organs, soil, puddle water and drainage water were sampled for analysis. Wheat plants were collected before and after the artificial *Fusarium graminearum* infection, and at the time of harvest, whereas maize

plants were only collected at the time of harvest. To determine the RALs distribution within the plant, one part of the collected plants from the time of harvest was separated and divided into kernels, leaves of stalks, spindles, husk leaves and stalks, and into leaves, kernels, spelts, stalks, stalks of the ear of maize and winter wheat, respectively. Soil samples from within the study field where regularly collected at randomly chosen sites at depths of 0-10 cm, 10-20 cm and 20-40 cm. One composite sample consisted of 10 to 20 sub-samples. A detailed description of the sampling procedure for soil matrices is described in Hartmann et al. *(20)*. Puddle water was sampled manually (September 2, 2005) at the time of heavy *Fusarium graminearum* infection on the wheat field after a heavy rain event. Drainage water samples were taken flow proportionally at a sampling rate of 200 mL per several hundred to several thousand liters (Table S2), depending on the season and cultivation. Five subsamples of 200 mL were combined to one composite sample. Sample treatment and analytical procedures for solid and aqueous samples were performed as described in Hartmann et al. *(21,22)*.

4.3 Results and discussion

ZON was the only compound among the RALs that was regularly detected in the investigated samples, including crop organs, soil, puddle water and drainage water. Its metabolites were detected at no time in any of these samples. The observed conversion of α-ZOL and β-zearalenol back to ZON in stored aqueous samples (data not shown) is in line with their absence in the investigated samples. Therefore the following section will be limited to ZON only. Results obtained for both plots (Figure 4.1) were basically similar, although drainage water loads were lower at the lower plot (Figure 4.1, B) because of reduced water flow. A water mass balance over the investigated period from June 2005 to August 2007 including precipitation, evapotranspiration and drainage water discharge showed that 73% and 44% of the upper and lower plot, respectively, were drained. For reasons of clarity, we will in the following focus on the upper plot (Figure 4.1, A). For comparison, corresponding data for the lower plot are given in the SI (SI, Figure S3).

4.3.1 ZON in total plants

Wheat and maize plants were sampled at different dates before and at harvest (Figure 4.2A). Artificial infection of the wheat fields in June 9, 2005 and May 25, 2007

led to visual *Fusarium graminearum* infestation of 74 and 80%, respectively. Figure 4.2A shows that ZON levels in both wheat cultivation periods increased with

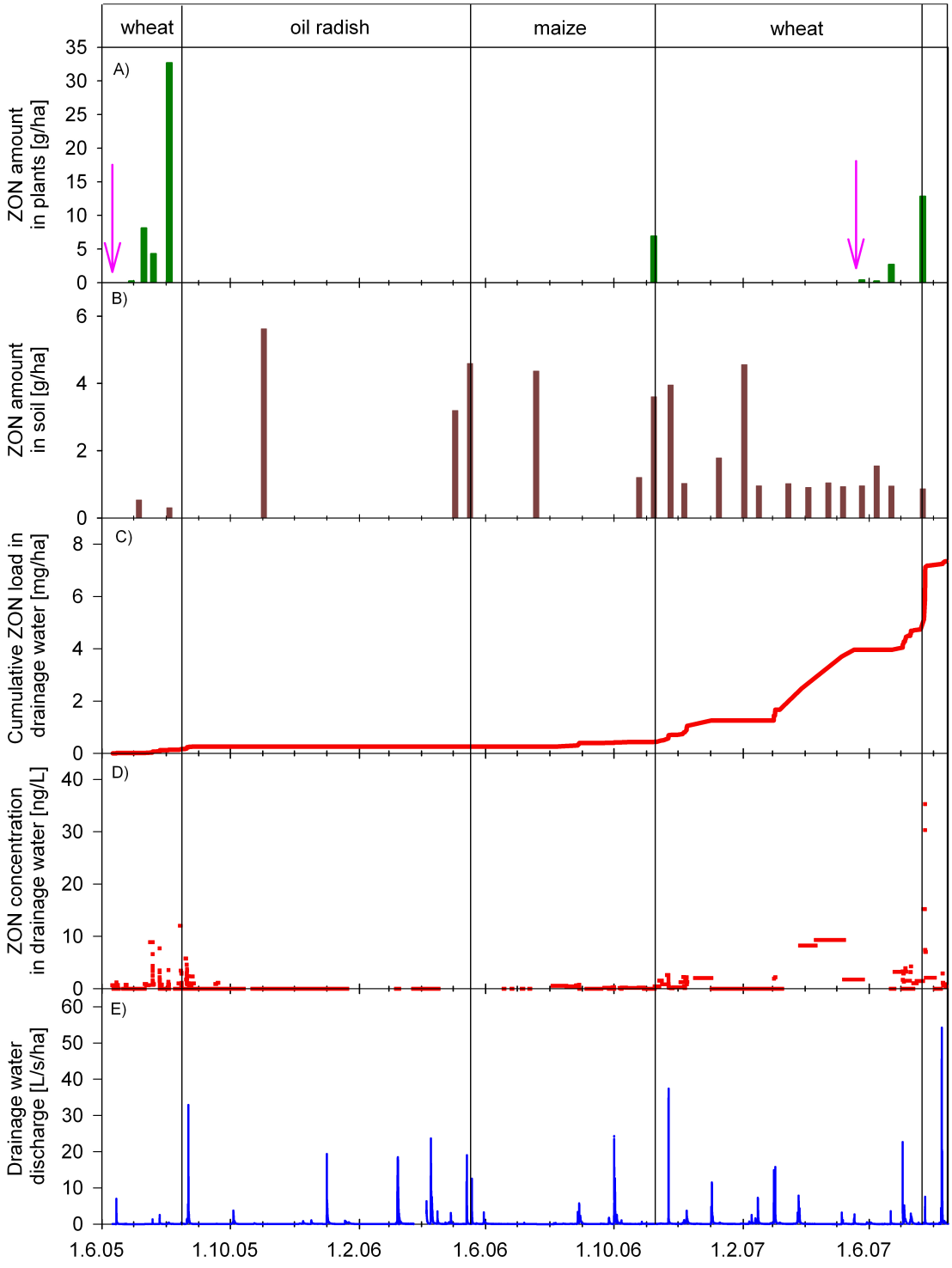

Figure 4.2: Occurrence of zearalenone (ZON) on *Fusarium graminearum* infested wheat and maize fields over a two year crop rotation on the upper plot. A) ZON amounts in the cultivated plants, B) ZON amounts in the topsoil (0-10cm), C) ZON loads emitted via drainage water, D) ZON concentrations in the drainage water and E) drainage water discharge. Individual crop periods (from sowing to harvest) are indicated by black vertical lines. Pink arrows indicate the time of artificial *Fusarium graminearum* infestation of the wheat field.

progressing plant maturity between the artificial infection and the time of harvest up to 33 and 13 g/ha in 2005 and 2007, respectively. However, absolute numbers at harvest differed, which mirrors the natural variability caused by, e.g., varying climatic conditions and fungi virulence. Moreover, it illustrates that the degree of infestation as determined by visual inspection does not necessarily correspond with total ZON amounts.

Although maize plants were not artificially infected with *Fusarium graminearum* spores, infection was visible at 21 and 53% of the stalks and husks, respectively, at the time of harvest (November 8, 2006). The total ZON amount present in maize plants was 7 g/ha. Also for maize, absolute numbers at the time of harvest differed as shown in the SI (Table S3).

4.3.2 ZON distribution in plant organs at harvest

ZON distribution in plant organs is important to know with regard to a) contamination of food and feed products and b) total amounts available on crop fields after harvest. ZON concentrations in the investigated wheat plant organs ranged from 0.1 to 16.6 (2005) and 0.1 to 7.1 $\mu g/g_{dry\ weight\ (dw)}$ (2007) (Table S3). Similar ZON levels were reported in Swiss feeding products *(23)*. Figure 4.3A shows the distribution of ZON in the wheat plant, calculated by the concentrations and the mass of each crop organ (Table S3, Figure 4.3). Thus it appears that 24 (2005) and 52% (2007) of the totally present ZON amount was associated with the kernels and removed with harvest. This pronounced inter-annual difference in wheat organ distribution again illustrates the natural variability caused by factors given above.

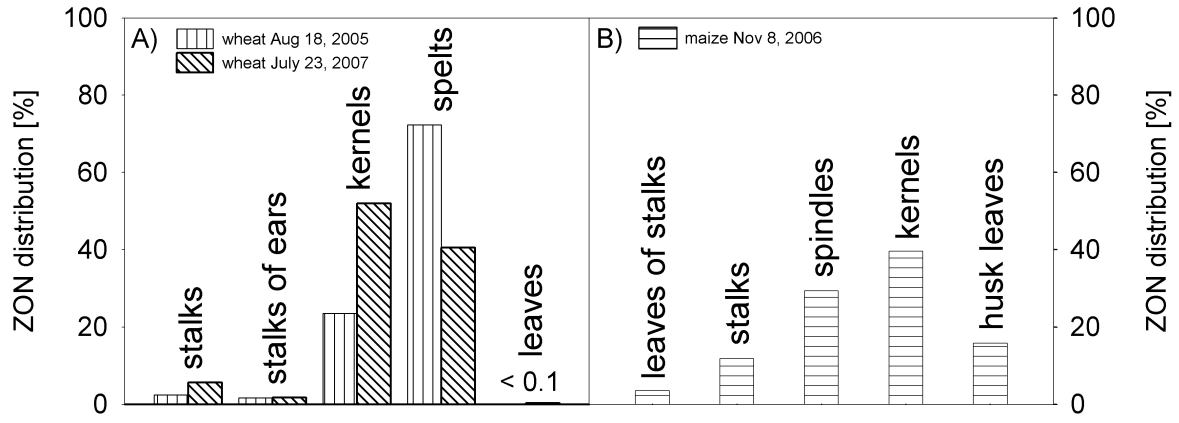

Figure 4.3: Zearalenone distribution within A) wheat and B) maize plants at the time of harvest.

ZON levels in the maize organs were between 0.1 and 1.4 µg/g_{dw} (Table S3). Figure 4.3B shows the ZON distribution within these crop organs, calculated as described above. In this case only 40% of the present ZON amount were associated with kernels and removed by harvest.

Consequently, at the time of harvest ZON amounts associated with plant debris were 25, 6, and 6 g/ha for wheat in 2005, maize in 2006 and wheat in 2007, respectively. These leftovers thus represent a major input source of ZON to soil. The degree of plant debris decomposition controls *Fusarium graminearum* survival, further ZON production, and release of incorporated ZON. Although degradation of ZON was only described in soil *(18)*, it probably also takes place in remaining plant debris. These processes make it difficult to quantify the ZON input to the soil at a given time.

4.3.3 ZON in soil

In general ZON levels in the topsoil (0-10 cm) varied between not detectable and 3.8 ng/g_{dw} (Table S4), corresponding to amounts between 0 and 5.6 g/ha (Figure 4.2B). Due to the continuous ZON emission from either *Fusarium graminearum* infested maize or wheat plants and/or plant debris on the soil surface, no clear concentration trend (Table S4) in the topsoil could be observed. The increase of the ZON amounts between July and November 2005 (Figure 4.2B) indicates that wheat plant debris after harvest (Figure 4.2A) was a major input to the topsoil. Between November 2005 and October 2006, a slight decrease (Figure 4.2B) of the ZON amount in the topsoil could be observed, which was probably caused by the lack of *Fusarium graminearum* infested crops and degradation of the already present ZON in the wheat plant debris. After maize harvest in November 2006 ZON levels in the topsoil were elevated, but decreased again during the following wheat period. Overall, it can be assumed that the continuously emitted ZON from the infected plants and its debris were at least partially compensated by dissipation. Reported dissipation half lives in soil for ZON were 6 to 11 days *(18)*. Similar degradation half lives of 7 to 30 days are given in the environmental assessment for α-ZOL which is in the US widely used as a growth promoter *(24)*. Therefore, a direct correlation of ZON amounts in crops and topsoil was not discernable and is probably not to be expected either.

In September 2005, ZON was quantified in manually collected puddle water (C_w) at 250 ng/L. Simultaneously, the ZON concentration in the topsoil (C_s) (0-10 cm) was

3750 ng/kg. From these data an apparent distribution coefficient (D) of 15 L/kg can be calculated ($D = C_s / C_w$). This distribution coefficient nicely corresponds with D numbers of 46 and 34 L/kg, obtained in sorption batch experiments after 1.5 and 3 hours contact time between water and soil phases. Considering the fact that this puddle water was in contact with only the top level of the topsoil, and that the sorption equilibrium time of 10 days was not reached (data not shown), the equilibrium distribution coefficient (K_d) between soil and puddle water is likely to be even higher. Indeed, the K_d of topsoil with an organic carbon fraction (f_{oc}) of 0.02 obtained in batch sorption experiments was 180 L/kg. Applying the relationship $K_d = K_{oc} * f_{oc}$, this results in a log K_{oc} of 4.0 was obtained, which is very close to the one calculated by Mortensen et al. *(18)* (Table 1). However, this presupposes that the organic carbon fraction is the only sorbent for ZON in the soil. Indeed, K_d-numbers of field soils from various depths (data not shown) suggest that the organic matter was at least the dominating sorbent, as it is known for many other organic chemicals *(25)*. Corresponding to the decrease of the organic carbon fraction with soil depth, K_d-values (data not shown), and ZON concentrations (Table S4) decreased as well.

4.3.4 ZON in drainage water

In general two stages could be distinguished in the ZON elution dynamics: 1) At certain times, especially during wheat and maize cultivation periods, ZON concentrations in the drainage water were in correlation with the drainage water discharge (“first flush” effect). This was already nicely visualized for a single discharge event in Hartmann et al. *(21).* Together with the considerable sorption behavior described above, this leads to the conclusion that ZON mission was mainly driven by preferential flow via macropores, 2) In periods with no or little precipitation, a permanent base flow of some mL/s drainage water was measured. During this time ZON levels were almost always below the detection limit of 0.6 ng/L. In the following ZON emission via drainage water will be discussed in a chronological order, subdivided in the four different cultivation periods (Figure 4.2).

Wheat cultivation from June 1 to August 18, 2005: During this period, drainage water contained ZON in concentrations up to 12.0 ng/L. At this initial state of the field experiment, the presence of ZON was solely caused by rainwater wash off from ZON contaminated wheat plants (Figure 4.2A). Although the present amounts in the wheat

plants were high (compared to later), the few drainage water discharge events (Figure 4.2E) led to total ZON emission of only 0.2 mg/ha.

Oil radish cultivation from August 18, 2005 to June 15, 2006: After wheat harvest only one more drainage water discharge event (Figure 4.2E) resulted in measurable ZON concentrations that probably originated from *Fusarium graminearum* infested wheat plant debris on the soil surface. The following drainage water discharge events during the oil radish cultivation did not lead to any further ZON emission (Figure 4.2D), although the ZON amount in the topsoil in November 3, 2005 was 6 g/ha (Figure 4.2B), which was the highest level detected throughout the whole field study. This again illustrates that strong sorption processes made the soil-bound ZON unavailable to aqueous elution. It can also be assumed that the wheat plant debris decomposed with time, and therewith the substrate for *Fusarium graminearum* fungi growth vanished and ZON production decreased. Consequently, emitted ZON amounts during oil radish cultivation were only 0.1 mg/ha (Figure 4.2C).

Maize cultivation between June 15 and Nov 8, 2006: Similar observations as with the preceding oil radish cultivation were made during this period. Note that single drainage water samples represented larger discharge volumes, which caused a general decrease in quantified ZON concentrations and a lower temporal resolution. Although total ZON amounts in the maize plants were substantially lower than in the wheat plants in 2005 (Figure 4.2A), with 0.2 mg/ha (Figure 4.2C), the total emitted ZON load over this cultivation period was very similar to the one of wheat in 2005.

Winter wheat cultivation from Nov 8, 2006 to July 23, 2007: Right after maize harvest in November 2006, the first drainage water discharge event still contained detectable concentrations of ZON due to contaminated maize plant debris on the topsoil. During this period, cumulative ZON loads in the drainage water were higher than during and after the wheat cultivation in 2005 (Figure 4.2C), even though ZON amounts associated with the respective plant debris were substantially lower (see above). This is probably caused by less frequent drainage water discharge events after harvest in 2005 than in 2007 (Figure 4.2E), and because wheat plant debris decomposed more quickly than the larger maize plant debris. Therefore ZON production and emission via drainage water continued until May 2007, although at this time the following wheat plants were not yet contaminated with ZON (Figure 4.2A). Later on, however, sources of ZON doubled, because the freshly *Fusarium graminearum* infested wheat crop added to loads of the ZON contaminated maize remnants. Together with the

overall favorable *Fusarium graminearum* conditions in summer 2007, the regular rainfall and the elevated drainage water discharge, this situation caused the highest ZON emission loads of around 4.3 mg/ha during this cultivation period.

In conclusion, ZON concentrations in the drainage water were mostly in the low ng/L range throughout the two year field study, with occasional peak concentrations up to 35.0 ng/L. These concentrations are comparable to reported 17β-estradiol levels from manure treated grassland fields *(26)*. As a result of the sampling strategy where each drainage water sample was composed of five subsamples, representing several hundred to thousand liters discharge, absolute peak concentrations might however not have been covered.

On the basis of the ZON amounts in the plant debris after harvest and cumulative ZON loads emitted via drainage water in the following cultivation period, ZON fractions eluting via drainage water over individual cultivation periods can be calculated. These were 0.001% for the oil radish and maize cultivation period (August 18, 2005 to November 8 2006), 0.070% for the second wheat cultivation period (November 8, 2006 to July 23, 2007) and 0.040% for the investigated period after wheat harvest in 2007 (July 23 to August 15, 2007). These fractions were considerably lower than the 1.2% reported for deoxynivalenol *(27)*, another *Fusarium* mycotoxin which we monitored simultaneously from July 9 to August 12, 2007.

4.3.5 Environmental relevance

Our data clearly show that soil is the primary recipient of ZON emitted from *Fusarium graminearum* infested wheat and maize fields. Unfortunately, our knowledge about the consequences of endocrine disruptors present in soil is still very limited. ZON concentrations in the drainage water were similar to 17β-estradiol levels found in surface waters *(28)*. Due to the large dilution of drainage water in surface waters, ZON concentrations will usually drop below detection limit and probably environmentally critical levels, although little is known about ecotoxicological consequences of pulsed exposures. In several hundred investigated surface water samples taken throughout the midlands of Switzerland *(20)*, ZON was detected in three cases only *(27)*. Still, in small creeks mainly fed by agricultural runoff, ZON might contribute substantially to the total estrogenicity of such water in case of

Fusarium graminearum occurrence. In the future, we suggest to extend the search for other mycotoxins acting as aquatic micropollutants *(27)*.

4.3.6 Supporting information available (see appendix)

Figure S1 shows chemical structures of the RALs, Figure S2 the precipitation and ground water level of the field site, Figure S3 the occurrence of zearalenone (ZON) on *Fusarium graminearum* infested wheat and maize fields over a two year crop rotation on the lower plot. Table S1 lists detailed soil parameters, Table S2 the drainage water sampling frequency, Table S3 the ZON distribution within crop organs of wheat and maize at the time of harvest, and Table S4 the ZON concentrations in the soil of the field site. This information is available free of charge via the Internet at http://pubs.acs.org.

Acknowledgments

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Chapter 5: Zearalenone sorption to soil

Sorption of Zearalenone on Soils and Selected Model Sorbents

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Manuskript in preparation

Abstract

The sorption of the estrogenic mycotoxin zearalenone (ZON) was investigated in various soils and to three selected model sorbents, in humic acid, goethite and montmorillonite. These represent organic carbon, metal oxides and clay minerals, the most important constituents in agricultural soils in temperate climate. Sorption data on soils indicate that the organic carbon was the most sorptive soil constituent with an organic carbon-normalized distribution coefficient log K_{oc} of 3.52 L/kg. This finding was corroborated by the sorption isotherms on the selected models sorbents. At pH=4 the Freundlich coefficients (log K_F) were 5.06, 2.85 and 2.20 $(ng/L)/(ng/kg)^n$ for humic acid, goethite and montmorillonite, respectively, reflecting the relative sorptivity of possibly present soil constituents.

5.1 Introduction

Zearalenone (ZON, Figure 5.1) is a naturally occurring endocrine disrupting chemical, produced by *Fusarium* fungi growing on a variety of cereals such as maize and wheat. Due to its high estrogenic activity *(1,2)*, ZON has been extensively studied in food and feed context around the world *(3,4)*. In a field study *(5)* we showed that *Fusarium* infected wheat and maize fields were important ZON sources for the environment. ZON concentrations in the soil of our field site reached up to several ng per gram dry weight and only a minor ZON fraction was emitted via drainage water *(5).* The ZON elution dynamic from the field site described in earlier publications *(5,6)* led to the conclusion that ZON emission was mainly driven by preferential flow via macropores and that considerable sorption to the soil took place. This assumption seems reasonable in light of a calculated log K_{oc} of 3.89 L/kg *(7,)* showing that ZON exhibits physical-chemical properties which cause sorption and retention in soil systems, in particular sorption to organic carbon *(8).* So far, sorption processes of ZON to soils and its constituents are not described in the literature.

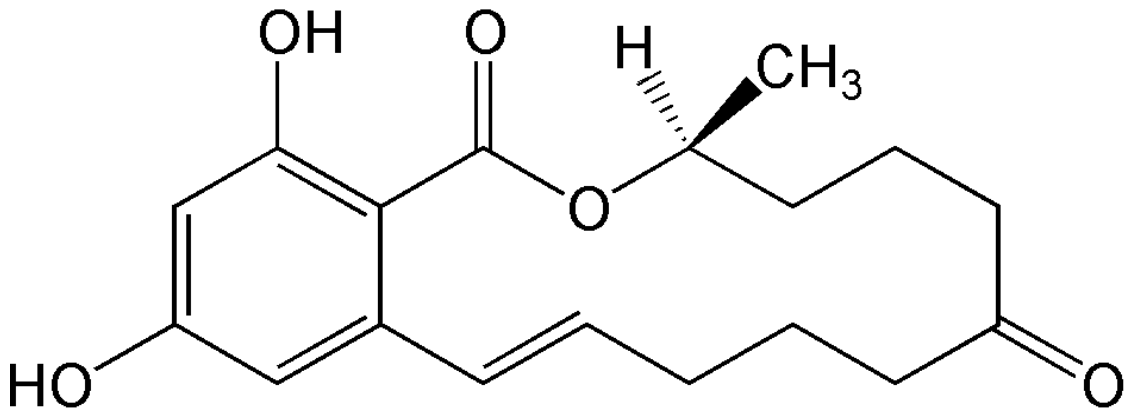

Figure 5.1: Chemical structure of zearalenone

Sorption processes depend on the chemical properties of both the analyte and the sorbent. ZON is an ionisable organic compound: The pK_a values of the phenolic moieties of the two resorcyclic acid lactones ZON and its derivative α-zearalanol are estimated to be between 7.6 *(9)* and 8.4 *(10)* (pK_{a1}) at position 4 (Figure 5.1), and 11.4 *(10)* (pK_{a2}) at position 2 (Figure 5.1). Hence, ZON sorption also depends on the ambient pH, due to (partial) deprotonation at position 2 and 4 under basic conditions. In the case of neutral ZON two general interactions with surfaces are possible: 1) non-specific interactions such as van der Waals (vdW) and dipole-dipole forces 2) specific interactions of the sorbates functional groups such as H-bonding. ZON is

classified as bi-polar as it has H-acceptor (both keto groups at positions 6' and 11') and H-donor (both hydroxy groups at positions 2 and 4) capabilities *(8)*. At pH levels around and above pK_{a2}, the deprotonated hydroxy group at position 2 together with the partially negative charged keto of the ester group at position 11' can probably form complexes with oxides such as Fe or Al *(8)*.

The extent to which specific interactions may be formed with ZON depends on the chemical characteristics of the major soil constituents, namely humic acids, iron oxides (e.g. goethite), and clay minerals (e.g. montmorillonite), respectively. Humic acid consists of numerous oxygen-containing functional groups and has a high aromaticity. As a result of these properties, H-bonding interactions with organic compounds are possible. Reported pK_a values of Leonardite humic acid were 4 and 6 for the carboxylic acids, 8 for the amino acids and 10 for the phenols *(11)*. Therefore additionally repulsive electrostatic interactions may occur at elevated pH. In general sorption of organic compounds to humic acids includes unspecific hydrophobic partitioning into the bulk material (absorption) and specific interactions with its functional groups *(8)*. Goethite is a hydrous oxide of iron with the chemical formula α-FeO*OH. It has a point of zero charge (PZC) of around 8, which implies that at an ambient pH below 8 its surface is positively and above 8 negatively charged *(12)*. At ambient pH below and around PZC no electrostatic interactions with ZON are possible due to the negative charge of the goethite and the neutral speciation of ZON, whereas at ambient pH above PZC electrostatic repulsion is to be expected. Hydroxygroups and Fe species at its surface allow H-bonding and complexation with keto- and hydroxygroups as shown, e.g., for oxytetracycline *(13)*. Montmorillonite is a 2:1 layered aluminous clay mineral with a permanent negative charge on its planar innerlayer surfaces due to isomorphic substitution in the clay mineral structure. This charge imbalance is offset by exchangeable cations, depending on the ambient solution *(14)*. Hydroxylgroups associated with Al at the edges are protonated at pH values below 8-9 and become deprotonated with increasing pH *(15)*. Therefore at ambient pH above 8-9, electrostatic interactions are possible due to the positive charge at the montmorillonite edges and the deprotonated ZON.

To date no data are available for ZON sorption on organic carbon and on metal oxides. In contrast, ZON sorption on organo-clays is well investigated. However these publications *(9,16,17)* investigated organo-clays as supplements in ZON contaminated feed for ZON sorption in the intestinal tract of animals. Moreover,

because organo-clays are very different to the clays present in soils, these data are difficult to transfer to environmental conditions. Also information about phenomenological ZON sorption data to soils is very limited. Mortensen et al. *(7)* described a ZON dissipation rate of several days in soils. However, in their work sorption and degradation processes were not further distinguished.
The goal of this work was to quantify ZON sorption in soils. Therefore ZON sorptivity on various soils was correlated with their physical and chemical composition to identify the most sorptive soil constituent. To support the conclusions on the basis of this approach, ZON sorption was investigated at different pHs on three model sorbents humic acid, goethite and montmorillonite which represent the organic carbon, the iron oxide and the clay mineral fractions in soils.

5.2 Method and Materials

5.2.1 Sorbents

Soils (Table 5.1) were taken from the earlier field study which is described in Hartmann et al. *(5)* and from 11 stations from the Swiss soil monitoring network (NABO) *(18)*. Before use, the soils were dried at 40℃ until weight constancy was achieved, ground and sieved to 0.5 mm using a ZM1 centrifuge mill (Retsch GmbH, Germany).
Humic acid (Leonardite, International humic substances society, CO) was attached to non-porous Aerosil OX50 silica in our laboratory according to the method described in Koopal et al. *(19)*. In brief, the immobilisation of humic acid on silica involved the following steps: A) pre-treating of the silica, B) immobilization of the humic acid on the silica and C) end-capping of the remaining free amine groups on the silica. The organic carbon fraction of the resulting humic acid-silica sorbent was 2.03%. The humic acid was permanently immobilized on the silica and no humic acid was released to the aqueous solution *(19)*. In total, around 40% of the charged silica groups were involved in the immobilization step *(19)*. Goethite (32 m^2/g, Bayferrox 910) was purchased from Lanxess (Lanxess, Germany GmbH) and Na-rich montmorillonite (40 m^2/g, Swy-2) from the clay minerals society (West Lafaette, IN). Both sorbents were used as received. Their organic carbon fractions (f_{oc}) as determined by elementary analysis were 0.02% and 0.05% for goethite and montmorillonite, respectively.

Table 5.1: Soil parameters

Soil	Sampling depth [cm]	pH ($CaCl_2$)	C_{org} [%]	Clay [%]	Silt [%]	Sand [%]
Reckenholz [a]						
Ahp	0 – 20	6.8	2.0	30.6	39.4	30.0
Bg	20 – 35	7.0	2.6	44.2	28.9	26.9
Bgg	35 – 50	7.0	0.9	30.0	38.2	31.8
Bcgg	50 – 70	7.7	0.2	18.2	49.6	32.2
Cgg	70 – 90	7.8	0.1	13.2	66.3	20.5
Nabo [b]						
Nabo 3	0 - 20	6.3	1.0	15	16	69
Nabo 9	0 - 20	5.5	1.1	17	34	49
Nabo 15	0 - 20	6.1	18.5	48	30	22
Nabo 28	0 - 20	5.3	1.8	14	34	52
Nabo 31	0 - 20	5.9	2.5	21	18	61
Nabo 38	0 - 20	5.2	2.0	28	23	49
Nabo 44	0 - 20	5.9	1.9	14	33	53
Nabo 56	0 - 20	4.7	7.1	18	52	30
Nabo 65	0 - 20	6.0	1.5	16	67	18
Nabo 78	0 - 20.	6.6	1.1	16	38	46
Nabo 80	0 - 20	5.4	3.6	26	36	38

[a] Data collected at Agroscope Reckenholz-Tänikon ART, Reckenholz, 8046 Zürich, CH
[b] Data from the Swiss soil monitoring network *(18)*

5.2.2 Sorption experiments

The time to reach phase distribution equilibrium was determined for all sorbents with preliminary tests. Soil isotherm sorption experiments were accomplished for five soil horizons from the local field site and three NABO soils, namely No. 15, 56 and 80 with batch experiments following the procedure described in the OECD guideline 106 *(20)*. The ionic strength of the aqueous phase (water was purified by a Milli-Q system from Millipore, Switzerland) was adjusted with 0.1 M $CaCl_2$, and 0.2 g NaN_3 per litre suspension was added for sterilization. Batch experiments were conducted in Duran Schott bottles (Sigma-Aldrich, Switzerland) with a water to soil ratio of 100 ml/g, at 20℃ and daylight conditions. Seven ZON (Figure 5,1 , [CAS-No. 17924-92-4] (≥99%), purchased from Sigma, Switzerland) concentration levels in two replicates between 100 ng/L und 100 µg/L were chosen. Before ZON was spiked, the water/soil suspension was equilibrated for 24 hours. Bottles were shaken on an orbital horizontal shaker for the respective equilibration time. Sorption isotherms with the field soils were conducted without adjusting the pH. The pH was at around 6.5 and did vary less than 0.5 units from the beginning to the end of the experiment. Control experiments without addition of soil were performed to quantify possible losses of ZON due to sorption to the glass vial or degradation. According to these results, such

losses could be neglected. Similarly, the sorption of ZON from aqueous solution to several other NABO soils was investigated with one single concentration, assuming sorption linearity (see below).

Sorption isotherms of ZON to humic acid, goethite and montmorillonite were determined as described above for soil with some adaptions: A) instead of 0.1M $CaCl_2$ (as described in the OECD guideline 106 *(20)*), 0.1M NaCl was used to adjust ionic strength, to avoid sorbent clustering due to Ca^{2+} ions, B) no NaN_3 was added, C) the water to sorbent ratio was 500 ml/g for goethite and montmorillonite and 140'000 ml/g for humic acid and D) experiments were conducted at pH=4 and 10, which were adjusted with 0.1M HCl and 0.1M NaOH. The pH was controlled at the end of the experiments and remained stable within 0.5 pH units. Because only 40% of the charged groups on the silica surface were covered by humic acid *(19)*, a sorption isotherm on silica was performed for possible data correction. ZON sorption to silica was negligible, although the silica used was not end-capped, which is assumed having an even lower sorption capacity than the pure silica.

After equilibration time the water/solid suspension was filtrated with a glass fibre filter (pore size 1.2um, Millipore, Volketswil, Switzerland). The filtrate was then spiked with the internal standard, concentrated with solid phase extraction and further processed as described for aqueous samples in Hartmann et al. *(6)*. Depending on the concentration level different fractions of the filtrate were concentrated. The highest concentration was measured directly. Chemical analysis was performed as described in Hartmann et al. *(6)*.

5.2.3 ZON extraction

After completion of the sorption experiment, ZON analysis was performed for soils at the initial concentration of 300 ng/L to determine non-extractable residues. The soil was dried and then extracted with Soxhlet using MeOH (Scharlau, Spain) as solvent. The extract was then handled and analyzed as described in Hartmann et al. *(21)*.

5.3 Results and discussion

5.3.1 Soil sorption and extractability

Isotherm data from the five soil horizons and the three NABO (Nr. 15, 56, 80) were fitted with the Freundlich equation. The resulting parameters are given in table 5.2.

Sorption equilibrium was reached after 144 hours. Measured pH in drainage water *(6)* and in the topsoil *(5)* of the earlier conducted field study *(5)* was 6.5. Depending on the pK_{a1} value taken as basis of calculation at that pH between 1 and 8% of the ZON was present in the negative and the rest in the uncharged form.

Table 5.2: Freundlich parameters

Sorbent	log K_f [a] [(ng/L)/(ng/kg)^n]	n [b]	r^2 [c]
Ahp	2.22	1.01	0.99
Bg	2.16	1.05	0.99
Bgg	2.40	0.92	0.99
BCgg	2.18	0.96	0.99
Cgg	2.37	0.90	0.99
Nabo 15	2.77	1.14	0.98
Nabo 56	1.53	1.18	1.00
Nabo 80	1.42	1.23	1.00
Humic acid pH=4	5.06	1.02	0.97
Goethite pH=4	2.85	1.05	0.92
Goethite pH=10	-1.30	2.09	0.99
Montmorillonite pH=4	2.20	1.24	0.93
Montmorillonite pH=10	2.91	1.24	0.99

[a] Freundlich constant
[b] Freundlich exponent
[c] Correlation coefficient

In Figure 5.2 the distribution coefficients (K_d) at the initial ZON concentration of 1000 ng/L are plotted against the f_{oc} of the soil horizons and all NABO soils. This is justified due to the linearity ($0.90 \leq n \leq 1.23$; Table 5.2) off all soil isotherms, and because for the additional NABO soils sorption data were available only at the mentioned ZON concentration. Within the field soils (circles), the measured K_d correlates strongly (r^2=0.94) with the f_{oc} and from the slope (3318 L/kg) a distribution coefficient between the water phase and the organic carbon (K_{oc}) log $K_{oc\ (field\ soil)}$ of 3.52 L/kg was calculated, which was close to the reported log K_{oc} of 3.89 L/kg *(7)* and for the strucutrally similar 17ß-estradiol *(22)*. This simple model fits well for the field soils, but does not describe the sorption behaviour of the NABO soils (Figure 5.2, filled circles). Individually calculated log K_{oc} ($K_{oc}=K_d/f_{oc}$) of all soils varied between 3.22 and 5.26 L/kg. These large differences in log K_{oc} values can be explained by: A) the organic carbon having different properties due to different origins or different agricultural use, B) the assumption that organic carbon was not the only sorbent for ZON in the soils, C) ageing effects and D) probably lower pH values (Table 5.1) of all NABO soils (expect the one with the highest K_{oc}). Such differences in K_{oc} values were already reported for other compounds in the past *(23,24)*. The good correlation within the

field soils can be explained by the assumption that the organic carbon of the five horizons included similarly structured components. As the linear regression was not forced through zero, a y-axis intercept of 138 L/kg resulted. This implies that the organic carbon seems to be not the only, but a very sorptive soil constituent as described for other organic compounds *(8)*. The ZON fractions ($F=K_{oc\ (field\ soil)}*f_{oc}/K_d$) sorbed to organic carbon were 32.8, 39.6, 16.6, 5.0 and 1.8% for Ahp, Bg, Bgg, BCgg and Cgg respectively. The rest of the ZON was sorbed to other soil constituents.

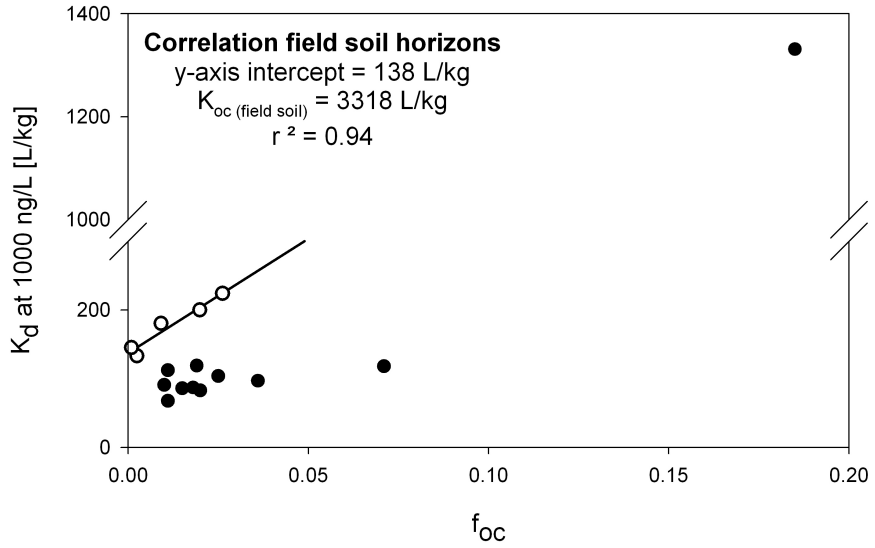

Figure 5.2: Circles: Samples from field soil horizons. Filled circles: NABO soils (0-20 cm). The line indicates the linear regression of the field soils distribution coefficient (K_d) at 1000 ng/L (initial concentration) versus the f_{oc}.

The extractability of ZON from the sorbed amount was tested only for the five field soil horizons (Ahp, Bg, Bgg, BCgg, Cgg). From the concentration in the water phases ZON amounts in the soils were determined by mass balance calculations. Thereof the extracted ZON fractions were 68.7, 73.4, 29.5, 4.9 and 4.1% and therefore higher or at least equal to the sorbed fraction to organic carbon. The linear fit of the non-extractable fractions showed a negative correlation (r^2=0.98) with increasing f_{oc}, which let to the conclusion that interactions between ZON and the organic carbon were more reversible than with other sorbents within the soil. This finding is in contrast to the general notion that non-extractable residues are positively correlated with soil organic matter *(25)*.

5.3.2 Sorption to model sorbents

Freundlich parameters for humic acid, goethite and montmorillonite are given in table 5.2. The ZON sorption isotherm (Figure 5.3a) at pH=4 on humic acid showed a linear and very strong sorption behaviour. Sorption on the pure silica, which was not covered by humic acid was negligible, thus no correction was necessary although only 40% of the charged silica groups were covered with humic acid *(19)*. A sorption isotherm at pH=10 could not be performed, because humic acid dissolved before sorption equilibration was reached. This did not happen at pH=4 *(19)*. Therefore no correction due to ZON sorption on dissolved organic carbon was necessary. The linearity implies that sorption was mainly driven by absorption into the bulk humic acid material. At pH=4, ZON was present in the neutral form, and therefore only vdW and H-bonding interactions were responsible for the strong sorption. From the f_{oc} (55%) of the pure humic acid and the log $K_{f(humic\ acid)}$ of 5.06 (ng/L)/(ng/kg)^n a log $K_{oc(humic\ acid)}$ of 5.32 L/kg (at pH=4) can be calculated, which is much higher than the log $K_{oc\ (field\ soil)}$ of 3.52 L/kg (at pH=6.8) calculated above. Part of these differences in the log K_{oc}'s might be explainable by the different pH at which the experiments were conducted. Such differences in K_{oc} values for different origins of the organic carbon were also reported for other organic chemicals such as sulfathiazole *(26)* and other organic compounds *(23,24)*.

The goethite sorption isotherm at pH=4 was inear (Figure 5.3b). Surface coverage at the highest concentration was less than 1%. Because again at pH=4 ZON was present in the uncharged form only vdW and H-bonding interactions caused sorption onto goethite. With a log $K_{f(goethite)}$ of 2.85 (ng/L)/(ng/kg)^n (Table 5.2) ZON sorption to goethite was two orders of magnitude lower than to humic acid but still quite high compared to other organic compounds such as 17β-estradiol *(27)* or several pesticides *(28)*. The sorption isotherm at pH=10 showed stronger non-linearity (Table 5.2), which cannot be explained so far. At the highest concentration, ZON distribution between the water phase and goethite was similar, although at pH=10 the negatively charged goethite surface should repulse the anionic ZON due electrostatic interactions as shown in Clausen et al. *(28)* for several pesticides.

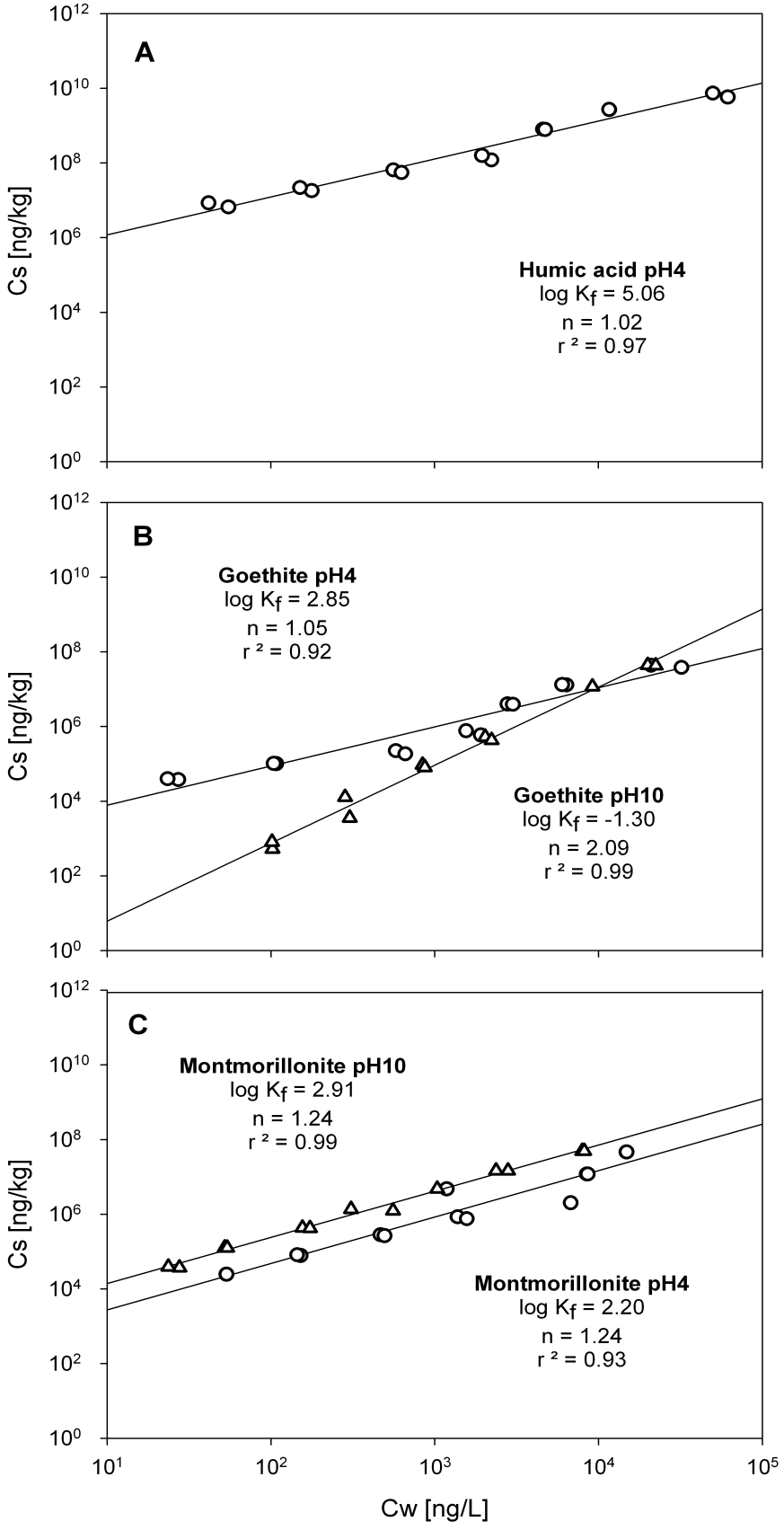

Figure 5.3: Sorption isotherms of ZON on humic acid (A), goethite (B) and montmorillonite (C), fitted by Freundlich equation.

The montmorillonite sorption isotherm (Figure 5.3c) at pH=4 was slightly non-linear (Table 5.2). The n>1 indicates promoted sorption with increasing ZON concentration in the aqueous phase. Surface coverage with ZON was around 1% at highest concentration. The log $K_{f(montmorillonite)}$ of 2.20 (ng/L)/(ng/kg)^n (Table 5.2) indicates an even weaker, but still considerable sorption of ZON on montmorillonite than on goethite. This number is much higher than reported for ZON sorption on unmodified base clay at neutral pH *(9)* but in a similar range as reported for 17β-estradiol *(27)*. Again at pH=4 only vdW and H-bonding interactions can be responsible for ZON sorption. At a first view it seems surprisingly that the log $K_{f(montmorillonite)}$ of 2.91 (ng/L)/(ng/kg)^n (Table 5.2) at pH=10 was higher than at pH=4 because of the permanent negative charge of the montmorillonite surface. The slight sorption increase at pH=10 might be explained by possible complexation of the deprotonated ZON with the Al at the montmorillonite edges as shown for oxytetracycline on goethite *(13)* or ligand exchange by replacing HO^- at its edges as shown for glyphosate *(29)*.

5.3.3 Sorption comparison between soils and model sorbents

Due to the fact that ZON in the drainage water and in the soil is mainly present in the neutral form, it is reasonable to compare the sorption on soils and the investigated model sorbents at the pH below pK_{a1}. As described above for soils, the organic carbon is a very sorptive soil constituent with a log $K_{oc\ (field\ soil)}$ of 3.52 L/kg (at pH=6.8). The log $K_{f\ (humic\ acid)}$ of 5.06 (ng/L)/(ng/kg)^n, the log $K_{f\ (goethite)}$ of 2.85 (ng/L)/(ng/kg)^n and the log $K_{f\ (montmorillonite)}$ of 2.20 (ng/L)/(ng/kg)^n (all at pH=4) support the findings, that for ZON the organic carbon was the most sorptive fraction in the soil. Further, ZON sorption to goethite and montmorillonite can be compared by surface-normalized K_f values. Due to similar surface areas of goethite and montmorillonite, such K_f values did not differ substantially from mass-normalized ones. Comparing the log $K_{oc\ (field\ soil)}$ with the log $K_{f\ (goetite)}$ and log $K_{f\ (montmorillonite)}$ two effects have to be taken in consideration: A) The K_f values of goethite and montmorillonite might be somewhat over estimated due to the small f_{oc} within these sorbents. However, total ZON amounts sorbed on the organic carbon calculated with a log K_{oc} of 3.94 L/kg (average of all investigated soils) were for goethite 0.1%

(pH=4) and for montmorillonite 2.4% (pH=4) and 0.3% (pH=10). B) In soil systems goethite and montmorillonite surface might not be fully available for ZON sorption.

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Chapter 6: Zearalenone in the agro-environmental context

Environmental Exposure to Estrogenic and Other Myco- & Phytotoxins

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Chimia, in press

Abstract

Zearalenone (ZON) is known as a very potent, naturally occurring estrogenic mycotoxin. It is one of the most prevalent mycotoxin produced as a secondary metabolite by *Fusarium* species growing on cereals such as wheat and corn. It has been studied extensively in food and feed products for decades but only rarely and somewhat by chance in the environment. We therefore elucidated its agro-environmental fate and behaviour by conducting a series of field studies and monitoring campaigns. Specifically, ZON was investigated in plants, soils and drainage waters from wheat and cornfields artificially infected with *Fusarium graminearum*. In addition, manure, sewage sludge and surface waters were analysed for ZON. Three main input pathways of ZON onto soil could be identified: 1) wash-off from *Fusarium*-infected plants (in the order of 100 mg/ha), 2) plant debris remaining on the soil after harvest (up to few g/ha), and 3) manure application (in the order of 100 mg/ha). Our results show that these input sources altogether caused the presence of several g/ha of ZON in topsoil. Compared to this, ZON emission by drainage water from *Fusarium*-infected fields was generally low, with maximum concentrations of 35 ng/L and total amounts of a few mg/ha. Due to dilution, ZON concentrations dropped below environmental relevance in larger surface water bodies. However in small catchments dominated by runoff from agricultural land, ZON might substantially contribute to the estrogenicity of such waters. Apart from ZON, other natural toxins monitored in this study, such as the mycotoxin deoxynivalenol or the estrogenic phytoestrogen formononetin, emitted to and occurred in surface waters at considerably higher amounts. To date their ecotoxicological effects are largely unknown.

6.1 Introduction

Mycotoxins are naturally occurring secondary metabolites of fungi growing on a variety of cereals. Among the most important mycotoxin producing fungi are *Fusarium* spp. They pose a severe economical threat, which in the US crop production of the 1990s led to losses of three billion US$ *(1).* No exact calculations are available for Europe, but in Switzerland both losses of crops and quality have increased in recent years *(2).* The extent of *Fusarium* infection and subsequent contamination by mycotoxins is determined by several factors: 1) climatic conditions, 2) crop rotation, 3) soil cultivation and 4) susceptibility of crop varieties *(3).*

Out of the many classes of mycotoxins produced by *Fusarium* fungi, resorcyclic acid lactones (RALs) are of particular concern with respect to endocrine disruption. The most prominent representative of the RALs is zearalenone (ZON). The estrogenic activity of ZON is comparable with those of natural estrogens *(4)* and is orders of magnitudes higher than those of many notorious synthetic endocrine disruptors such as bisphenol A, DDT or atrazine *(5,6).* The estrogenically most potent of all RALs, α-zearalanol (α-ZAL), is still used as growth promoter for ruminants in the US and Canada, but has been banned in the EU since 1985. In the past it has been shown that RALs can cause severe reproductive and infertility problems in husbandry animals *(7,8)* due to their high estrogenic potencies.

Although the occurrence of ZON has been studied extensively in food and feed stuff *(9,10),* only little is known about its environmental distribution and impact. Several publications reported the occurrence of ZON in surface waters *(11,12)* and as well as in- and effluents of waster water treatment plants (WWTP) *(11,13,14).* Concentrations ranged from not detected up to 60 ng/L for individual samples. In some cases also other RALs such as α-zearalenol (α-ZOL), α-ZAL and β-zearalanol (β-ZAL) were detected at similar levels as ZON. For comparison, numbers in the same order of magnitude are observed for natural steroids *(15,16).* From the limited data summarized in Bucheli et al. *(17),* RALs seem to appear in surface waters occasionally throughout the year. Unfortunately, none of the mentioned studies further investigated on their potential emission sources. Only Lagana et al. *(11)* suspected the presence of RALs in surface waters to be primary caused by cattle excretion of growth promoting residues. RALs data from other environmental

compartments are presently not available, although Mortensen et al. *(18)* developed an analytical method to quantify ZON in soils.

The goal of this work was to elucidate the environmental distribution of ZON. Thereby, our main focus was on the input from small grain cereal and corn production as this is where mycotoxins are initially produced and known to occur. To our knowledge, this is the first time the occurrence of ZON and its metabolites were investigated in such detail, and with a view to relate their presence in the environment to possible sources.

Furthermore we reveal that ZON stands as an example for a wider range of naturally occurring toxins. Given the diversity of agricultural production systems, many other compounds e.g. other mycotoxins like deoxynivalenol (DON), or phytoestrogens such as the isoflavones exhibit the potential to enter the environment. In particular, we argue that such natural toxins of this kind should be considered as aquatic micropollutants *(19,20).*

6.2 Experimental section

To elucidate the pathways of RALs into the environment, we developed analytical methods for aqueous (drainage water, river water, WWTP effluent) *(21)* and solid phases (soil, manure, sewage sludge, plant materials) *(22)* were developed. Using adequate extraction and concentrations steps, ZON, α-ZOL, β-zearalenol, α-ZAL, β-ZAL and zearalanone could be detected by LC-MS/MS in the low ng/L and ng/g range for aqueous and solid matrices, respectively.

Several complementary approaches were chosen to study the environmental distribution of ZON. First, we investigated the presence of ZON in plants and soil of wheat- and cornfields infected with a mixture of RALs producing *Fusarium graminearum* isolates, as well as its emission via drainage water. This field site is briefly described in Hartmann et al. *(21),* Erbs et al. *(23)* and Bucheli et al. *(20).* A more detailed description will be published elsewhere *(24).* Second, we assessed the ZON input manure application by analysing a range of manure samples from the Swiss soil monitoring network (NABO). Additional information about manure application practice allowed calculations of potential annual ZON loads *(22)* entering agricultural land. Third, ZON was monitored in digested sewage sludge, which integrated hydrophobic micropollutants from the respective WWTP catchments. Digested sludge samples were gathered from the existing monitoring network named

Observation of the Metabolism of the Anthroposphere (SEA) *(25)* and further selected WWTPs. In addition, certain waste water samples were also analysed. Detailed information about catchment area characteristics (rural, urban, separated or mixed sewer systems, etc.) of each WWTP facilitated the apportionment of ZON input sources *(22).* Fourth, surface water samples were gathered from two existing monitoring networks a) Office for Waste, Water, Energy, and Air of the Canton of Zürich (AWEL) and b) National Long-Term Surveillance of Swiss Rivers of the Swiss government (NADUF) *(20,24).* Sampling stations were chosen based on their orographically cumulated ratio of winter wheat area within their catchment to water discharge *(17,20).* Finally, air was sampled by a high volume air sampler before and during harvest time at the field site to monitor possible ZON emissions via airborne soil- and plant particles.

Other natural toxins suspected to act as aquatic micropollutants were included in the above described studies at certain times. Specifically, DON, the estrogenic isoflavones formononetin (FOR), biochanin A, daidzein, genistein, equol, as well as coumestrol were quantified in drainage and surface waters over the period of their main production in spring and summer of 2007, using the analytical method described in Bucheli et al. *(20)* and Erbs et al. *(23).*

6.3 Results and discussion

Figure 6.1 illustrates in a simplified manner the probable environmental and urban distribution of RALs as suggested by the results of our studies. Pathways and compartments of food and feed production have already been thoroughly investigated *(26)* and are basically understood. Therefore, they were not a part of this study. All samples gathered were analyzed for all RALs, but only ZON was detected regularly, Hence, the following discussion will be limited to ZON.

6.3.1 Input of ZON into the terrestrial environment

We investigated two potentially main input pathways of ZON into agricultural soils, i.e., wash off from the plant before harvest or from plant debris remaining on the soil after harvest, and manure application (Figure 6.1). During heavy rain events water puddles containing ZON in concentrations of several hundred ng/L *(24)* where formed on the wheat field. Since these puddles occurred before harvest at a time

where the plants were severly infected by *Fusarium graminearum* it is reasonable to assume

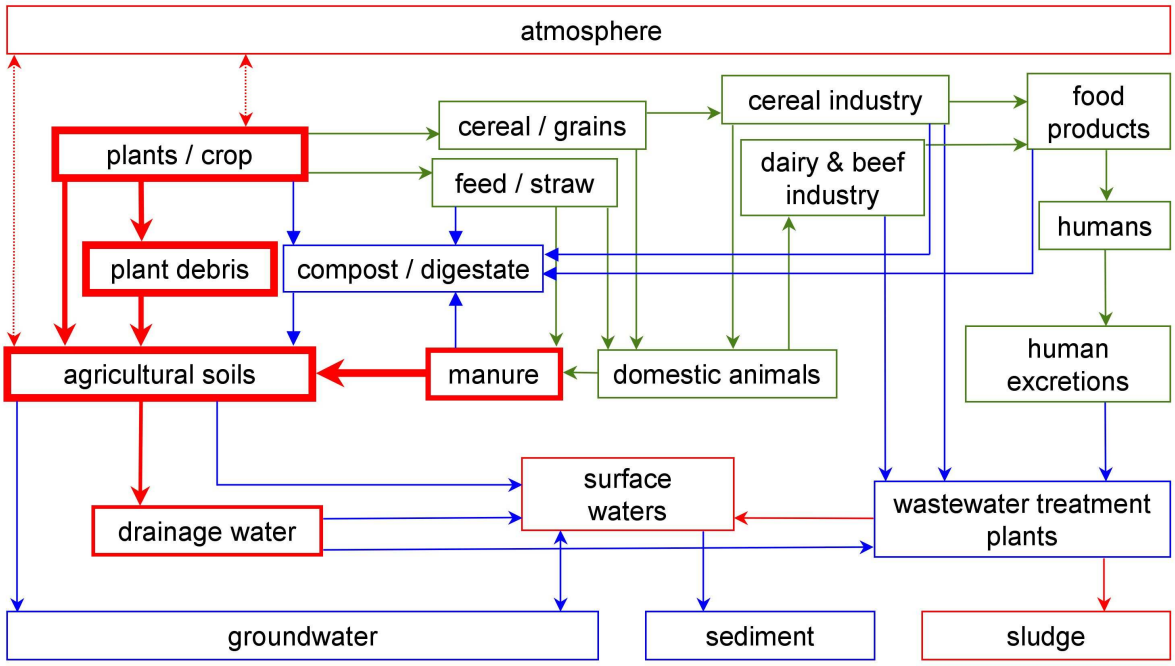

Figure 6.1: Suggested environmental distribution of RALs: Red coloured pathways and compartments: investigated (thickness of red lines reflects the relative importance); Red coloured dotted lines: investigated, ZON transfer does not occur; green coloured: not investigated because well investigated and understood; blue coloured: not investigated in this study.

that the quantified ZON was washed off the wheat plant directly by rainwater. Assuming a rain event of 30 mm and a ZON concentration of 250 ng/L in the puddle water, around 75 mg/ha ZON would have reached the soil surface. Based on two to four such rain events during the period where wheat plants were heavily infested by *Fusarium graminearum*, this translates to 150 and 300 mg ZON per ha. Direct wash off from *Fusarium* infested corn plants also takes place, but was not specifically investigated. The very high ZON concentrations ranging from 0.1 to 17 µg/g dry weight (dw) quantified here in several wheat and corn plant organs *(24)* suggest that *Fusarium* infested plant debris remaining on the field after harvest constitutes the more significant input source. During 1999 to 2005 Swiss feeding stuff contained ZON levels of 50 to 100 ng/g dw in 0 to 30% and 10 to 35% of the wheat and corn samples, respectively *(27).* Highest levels exceeded 400 ng/g dw which is within the observed levels in our field study. For other countries, levels up to 10 µg/g dw were reported in cereal grains and animal feed *(28).* Although concentrations varied strongly between plant organs, up to 15 g of ZON per hectare may be accomodated

by straw on the soil surface after harvest *(24).* Please note that the actual amount of ZON deposed on soil in this way depends very much on the agricultural practice.
At the same time, we detected ZON concentrations in the topsoil (0-10 cm) up to 4 ng/g dw, corresponding to almost 6 g/ha. Unfortunately very little is known about the fate of ZON in soils. A recent study estimated a half live time for ZON in Danish soils of 6 to 11 days, but did not differentiate between degradation and sequestration processes *(29).* Results from our own soil sorption experiments revealed considerable overall sorption coefficients (K_d) of 130-230 L/kg, with organic carbon as the dominating solid phase fraction (K_{oc} = 3318 L/kg_{oc}). Since the ZON concentrations reported in this study reflect a worst case situation in terms of *Fusarium* infection they may be inappropriate for up-scaling. However, they still represent realistic local scenarios where ZON amounts in this order of magnitude may occur.
Frequent presence of ZON in feed stuff (see above) lead to the excretion of substantial fractions of ZON in manure by husbandry animals *(30),* and becomes therefore another possibly relevant input source of ZON. Concentrations in manure samples were between 7 and 330 μg/kg dw corresponding to 50 - 150 mg/ha ZON reaching agricultural soils via manure application annualy *(22).* In comparison, 17β-estradiol concentrations in swine and cattle manure varied between 100 and 1215 μg/kg dw *(31)* and 0.8 and 30 μg/kg wet weight *(32).* These ZON loads are in the same range as those washed off from *Fusarium* infested cereals by rain (see above).
Although not investigated within the current project, the application of compost and digestate used as soil improver and fertilizer should be kept in mind as another potential input source of ZON (and other mycotoxins) to soil, because contaminated straw and residues from cereal processing may be used as input material. Estimates based on average to high concentrations of ZON these products, the assumption of the chemical stability of ZON during processing, and a standard fertilization of 70 kg P_2O_5 per hectare in the form of compost or digestate application, lead to a ZON load that could be comparable to those described above for other soil input pathways *(33).*
We did not detect ZON in any air sample sampled at our field site, not even the one covering the critical time of harvest during which a lot of dust was generated. Hence, outdoor occupational exposure with mycotoxins is probably less a problem compared with indoor workplaces on farms or other grain production sites *(34,35).*

6.3.2 Estrogenic and other mycotoxins and phytoestrogens as aquatic micropollutants

We investigated the emission of ZON via drainage water from *Fusarium* infested wheat and corn fields to assess its relative importance in comparison with other endocrine disrupting chemicals in surface waters *(36).* A detailed presentation and discussion of this emission study will be published elsewhere *(24).* Here, we focus on a limited period of time from our three years study. From July to August 2007, the ZON concentrations in drainage water were on average 3 ng/L with a maximum of 35 ng/L (Table 6.1) and the total ZON load emitted from the field site was 3 mg *(21,24)* during that period of time. Compared to the 15 g initially present on the field (see above), the fraction emitted via drainage water constituted only 0.02%.

Micropollutants such as 17β-estradiol *(37)* and sulfonamide antibiotics *(38)* were reported to emit from manure treated fields during rain events. It is reasonable to assume that this process takes place for ZON as well, since the aqueous solubilities of ZON and 17β-estradiol are comparable *(17).*

Table 6.1) Compilation of emission and river water data for ZON, DON, FOR and pesticides

	ZON	DON	FOR	Pesticides [a]
Emission studies (data per ha)				
period of investigation	Jul-Aug 07	Jul-Aug 07	Mar-Sept 07	season
amounts produced/applied	15 g	50 g	several kg	50 g – 1 kg
amounts emitted	3 mg	650 mg [b]	40 mg	3 mg – 56 g [c]
fraction emitted	0.02 %	1.3 % [b]	n.a.	0.0002 – 1.0 % [c]
average conc.	3 ng/L	560 ng/L [b]	189 ng/L	ng/L – ug/L
maximum conc.	35 ng/L	4.9 ug/L [b]	1.7 ug/L	ug/L – mg/L
Concentrations in river waters				
period of investigation	Apr 05–Oct 06, Jul 07	Jul–Aug 07	Mar–Sept 07	1999 – 2005 [d]
no. of analyses / no. of detects	several hundreds / 4	52 / 31 [b]	262 / 259	617 / 653 [d]
average conc. (of detects)	det.	8 ng/L [b]	10 ng/L	- [e]
maximum conc.	det.	22 ng/L [b]	132 ng/L	1.49 µg/L [d]

[a] Atrazine data. [b] ref [20]. [c] ref [40]. [d] ref [41]. [e] 327 of 653 analyses < 50 ng/L [41].
n.a. not available

In contrast to recent reports *(11,13,14),* ZON was not detected in any of the WWTP effluent samples analyzed here *(23).* However, we detected ZON below the quantification limit in about 25% of 87 individual sewage sludge samples and quantified it at several ng/g dw in two samples *(22).* Although these results show that

ZON occasionally occur in wastewater, its input via WWTP effluents into surface waters is probably negligible, especially in comparison with steroid hormones.
Out of several hundred surface water samples regularly taken between 2005 and 2007, ZON was detected below quantification limit in only four samples from summer 2007 *(20)* (Table 6.1).
Apart from ZON, several other natural toxins such as other mycotoxins or phytoestrogens could occur in drainage and surface waters as a consequence of the environmental distribution processes elucidated above. To test this hypothesis, we selected two model compounds and monitored these during the time of their main production in spring and summer 2007. DON was selected as a representative of other *Fusarium* mycotoxins *(20)* whereas FOR represents the estrogenic isoflavone present in legumes such as red clover *(39).* Table 6.1 compiles emission and surface water data obtained in our studies for ZON, DON and FOR. For comparison, emission data reported in the literature for pesticides - representing classical micropollutants – are included in Table 6.1 as well. The amounts of ZON and DON produced on *Fusarium* infested wheat fields are comparable with application rates of modern pesticides, which are used at amounts of a few dozen grams per hectare. The estimated amount of FOR on grassland was several orders of magnitudes higher than those of ZON and DON, and was even higher than usual application rates of pesticides. The differences in the amounts and fractions of ZON, DON and FOR emitted via drainage water can be explained by several factors, such as the availability at the plant surface for wash off, the aqueous solubility, and related to the former, the solid-aqueous phase distribution in soil. Overall, emitted amounts and fractions were within the range reported for pesticides such as atrazine, metolachlor and dimethenamid *(40).* DON and FOR concentrations of up to 22 ng/L detected in river waters in this study are comparable to the atrazine levels found in Swiss rivers [41], where 50% of all values ranged from not detected to 50 ng/L.
The ecotoxicological consequences of the occasional presence ZON, DON and FOR in the ng/L to µg/L range in aqueous environments remain to be elucidated. We assume that in most surface waters, ZON from agricultural runoff will be diluted to concentrations well below environmental relevance. However, in small water bodies receiving mainly runoff from wheat and corn fields and in case of *Fusarium graminearum* infection, ZON might contribute to the total estrogenicity. As for other mycotoxins and phytoestrogens, including DON and FOR, their ecotoxicological

significance is largely unknown, since they are normally considered a risk for food and feed only, and thus do not undergo ecotoxicity testing. It is known however that enniatins and beauvericins *(42)* as well as certain other trichothecenes *(43),* exhibit insecticidal effects. From the limited data currently available for FOR, we estimate its estrogenic effect to be somewhat similar to that of ZON.

Acknowledgements

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Chapter 7: *Fusarium* mycotoxins as aquatic micropollutants

Fusarium Mycotoxins: Overlooked Aquatic Micropollutants?

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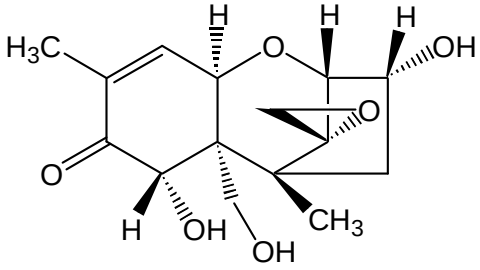
Abstract

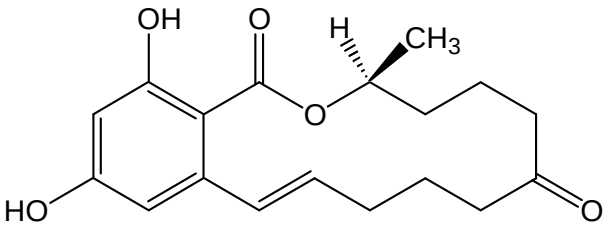
Deoxynivalenol and zearalenone are among the most prevalent toxins produced by *Fusarium* spp. They have been investigated in food and feed products for decades, but rarely in the environment. We therefore established solid phase extraction and LC-MS methods to quantify these mycotoxins at trace concentrations in aqueous natural samples. In a model emission study, we inoculated a winter wheat field with *F. graminearum* and subsequently monitored deoxynivalenol and zearalenone in its drainage water. Before, during, and after harvest in June and July 2007, these toxins were emitted in concentrations from 23 ng/L to 4.9 µg/L for deoxynivalenol, and from not detected to 35 ng/L for zearalenone. Simultaneously, in July and August 2007, deoxynivalenol was also detected in a number of Swiss rivers in concentrations up to 22 ng/L, and zearalenone was present in several river samples below the method quantification limit. Other mycotoxins might be emitted from *Fusarium*-infected fields as well, as some of them are produced in similar amounts as deoxynivalenol and zearalenone, and exhibit similar or even higher water solubility than deoxynivalenol. The ecotoxicological consequences of the presence of mycotoxins in surface waters remain to be elucidated.

7.1 Introduction

Mycotoxins are naturally occurring metabolites of fungal species growing on a wide variety of crops such as small grain cereals and maize. *Fusarium* spp*.* are among the most important toxigenic fungi (*1-4*), causing the cereal disease *Fusarium* head blight (FHB). Epidemics caused by FHB resulted in economic losses of US$ 2.7 billion in US wheat and barley production between 1998 and 2000 (*5*). The most prevalent mycotoxins produced by *Fusarium spp.* include deoxynivalenol and zearalenone (*1, 2*) (Figure 7.1). Zearalenone, in particular, is a potent natural estrogen (*6-9*).



Deoxynivalenol



Zearalenone

Figure 7.1: Structures of deoxynivalenol and zearalenone.

Owing to their considerable economical and health risk (*10*), the occurrence of mycotoxins in food and feed products has been studied extensively and for decades (*11, 12*). In contrast, very little is known about the distribution of *Fusarium* and other mycotoxins in the environment. Two soil column leaching experiments with fumonisins and aflatoxins (*13, 14*) showed some mobility of the quite stable and water soluble fumonisins. Mortensen et al. (*15*) studied the dissipation of ochratoxin A and zearalenone in soil and found apparent half-lives of 0.2 to one, and six to eleven days, respectively. Several recent publications reported the occasional occurrence of resorcyclic acid lactones in waste water treatment plant influents and effluents, as well as in river waters (*16-20*). Concentrations of zearalenone, and its derivatives α-zearalenol, α -zearalanol and β-zearalanol, ranged from not detected up to 60 ng/L. Their presence in the aquatic environment was suspected to be primarily caused by excretion of cattle treated with the growth promoting α-zearalanol (*16*), although such products are banned in the EU since 1985 (*21*). To our knowledge, no environmental data are available for other mycotoxins, including deoxynivalenol.

We have earlier hypothesized (*22*) and recently demonstrated (*23*) that zearalenone can be emitted into the aquatic environment via runoff from *F. graminearum*-infested agricultural fields. For the current study, we have extended our ongoing investigations on the environmental fate and behaviour of mycotoxins and include deoxynivalenol as one of the most prevalent *Fusarium* toxins worldwide (*1*). To do so, we first developed an analytical method for its quantification in aqueous samples in the low ng/L concentration range, using $^{13}C_{15}$-deoxynivalenol as internal standard. Deoxynivalenol and zearalenone were then quantified concomitantly in drainage water and in Swiss river water systems around the period of wheat harvest.

7.2 Methods and materials

7.2.1 Deoxynivalenol and zearalenone analysis

Deoxynivalenol and zearalenone were solid phase extracted from aqueous samples, followed by separation and detection with LC-MSMS. The internal standards $^{13}C_{15}$-deoxynivalenol (Biopure Referenzsubstanzen GmbH, Tulln, Austria) and D_6-zearalenone, prepared in our laboratory by base catalyzed hydrogen-deuterium exchange on native zearalenone (*24*), were added together as 50 µL of a 2 ng/µl methanol (MeOH) solution into the filtered (glass fibre filters, 1.2 µm) 1 L water samples, prior to extraction of deoxynivalenol and zearalenone with OASIS HLB (6cc, 200 mg) SPE cartridges (Waters Corp., Milford, MA). The internal standards thus largely compensated for losses during extraction and by ion suppression. The SPE cartridges were conditioned with 4 mL of MeOH, 4 mL of MeOH/Milli-Q water (50:50, v/v), and 4 mL of Milli-Q water, consecutively. Water samples were drawn by vacuum through the cartridges at a flow rate of 5 to 10 mL/min. The cartridges were subsequently washed with 5 mL Milli-Q water and dried by vacuum. Finally, the analytes were eluted with 4 mL of ethyl acetate into conical micro reaction vials and evaporated to dryness using a gentle nitrogen gas stream. The dried extracts were reconstituted in 400 µL of Milli-Q water/MeOH (90:10, v/v) and transferred into amber glass vials. The samples were stored at 4 ℃ and an alysed within 48 h.

LC–MS/MS was performed on a Varian 1200L LC–MS instrument (Varian Inc., Walnut Creek, CA). Analysis of zearalenone was performed as described Hartmann et al. (*23*). Deoxynivalenol was separated from other trichothecenes on a 50 mm × 2.0 mm i.d., 3 µm Polaris C18 A column (Varian Inc., Walnut Creek, CA) at room

temperature by applying the following elution gradient: 0 min, 5% B (95% A); 1 min, 5% B; 4 min, 30% B; 5 min, 100% B; 7 min, 100% B; 7.5 min, 5% B; 15 min, 5% B; with eluent A consisting of Milli-Q water/methanol (95/5, v/v) and eluent B of Milli-Q water/methanol (5/95, v/v). Both eluents were buffered with 5mM ammonium acetate (pH 6.8). The injection volume was 20 µL and the mobile phase flow rate was 0.25 mL/min, resulting in a retention time of 2.9 min for both deoxynivalenol and $^{13}C_{15}$-deoxynivalenol. Interface parameters of the LC–MS/MS were as follows: corona: -10 µA, shield voltage: −600V, drying gas (N_2, 99.5%) 225 ℃ and 1.23 bar, nebulizing gas (compressed air) 3.34 bar, vaporizer gas (N_2, 99.5%) 310 ℃ and 0.90 bar, housing temperature 50 ℃. Detection was performed in the (−)APCI mode using the following mass transition reactions: deoxynivalenol (355→265, 10 eV; 355→295, 10 eV; 295→265, 8 eV), $^{13}C_{15}$-deoxynivalenol (370→310, 10 eV; 370→280, 10 eV). The collision cell gas (Ar, 99.999%) pressure was 2.67 x 10^{-6} bar and the detector voltage was set to 1800V. Deoxynivalenol was quantified using calibration standards in Milli-Q water/MeOH (90:10, v/v), containing the internal standard. Data processing was carried out using the software Varian MS Workstation (v. 6.8, SP1). Analytical quality control parameters for deoxynivalenol in drainage and river water (ion suppression, absolute and relative recoveries, method precision method detection limits and instrument linearity) were determined as described for zearalenone (*23*).

7.2.2 Experimental fields

Two adjacent but separately drained 0.2 hectare plots close to our laboratories were selected as experimental fields. Both were equipped in 2004 with new drainage tubes and sampling shafts, which allowed the installation of drainage water flow meters and automated, flow proportional sampling (200 mL sample per 1000 L water, pooled to yield 1 L samples). This type of sampling allows quantifying pollutant loads in a given time period from measured concentrations and actual water flow. To study the potential production and runoff of mycotoxins from agricultural fields over a multi-year crop rotation, the following conditions favourable for *F. graminearum* infection and subsequent mycotoxin contamination were chosen: A winter wheat-maize rotation, cultivation of a wheat and maize variety (Levis, and Birko, respectively) susceptible to *F. graminearum* infection, no-till seeding resulting in maize residues on the soil surface and thus providing sites for survival of the fungus and a natural inoculum

source of *F. graminearum* infections, as well as artificial infection of wheat with conidia suspensions of *F. graminearum*. For enhanced throughput, the two plots were cultivated in an anticyclic manner with wheat or maize. Drainage water was monitored, sampled, and analysed from April 2005 until August 2007 for zearalenone, and from June 20 until July 30 2007 for deoxynivalenol. In the following, we will mainly present and discuss data gathered from a wheat field during the period of June 20 until July 30 2007.

7.2.3 Surface water monitoring

To elucidate the occurrence of the mycotoxins deoxynivalenol and zearalenone in Swiss surface waters, a range of observation sites from the monitoring programs of the Canton of Zurich (Office for Waste, Water, Energy, and Air; AWEL) and the Swiss government (National Long-Term Surveillance of Swiss Rivers; NADUF) were adapted to collect additional weekly (AWEL) and fortnightly (NADUF) integrated and flow proportional samples for mycotoxins analysis. The water bodies were selected based on the winter wheat and corn cultivation areas in their catchments. Specifically, monitoring sites located along the following rivers were chosen: Töss (at Rämismühle, and Freienstein), Kempt at Winterthur, Eulach at Wülflingen, Glatt (at Fällanden, Oberglatt and Rheinsfelden), Aabach at Mönchaltdorf, Aa at Niederuster, Thur at Andelfingen (all located in the canton of Zurich, and belonging to the AWEL monitoring program), Aare at Brugg, Reuss at Mellingen, Rhine at Rekingen (all canton Aargau), Klein-Emme at Littau (canton Luzerne), and Saane at Gümmenen (canton Berne) (all part of NADUF). Water samples were collected and analysed for deoxynivalenol from July 9 until August 12 2007 (calendar weeks 28 to 32, five samples per station in total) and for zearalenone from April 2005 to October 2006, and from July 9 to August 5 2007 (calendar weeks 28 to 31, four samples per station within this period). Deoxynivalenol loads were calculated from the quantified concentrations and the averaged daily water flows obtained from the AWEL and NADUF sampling station protocols.

7.3 Results and discussions

7.3.1 Deoxynivalenol and zearalenone analysis

This is the first study to develop and apply a method for quantification of deoxynivalenol in natural aqueous samples at the low ng/L concentration range. In particular, the use of $^{13}C_{15}$-deoxynivalenol as an internal standard ensured an accurate quantification of the target analyte, largely independent of interfering matrix compounds during ionization and losses occurring during sample preparation. Instrument linearity was tested and proved to be linear between 20 to 50'000 pg. Ion suppression for deoxynivalenol was 18 and 14%, absolute method recoveries 85 and 87%, relative recoveries 91 and 101% in drainage water, and river water, respectively. Method detection limits derived from three times the signal to noise ratio in environmental samples were 1.4 and 1.5 ng/L, and method precision as determined by multiple analysis (n=5) of spiked samples (5 and 25 ng/L) was 5 to 12% in drainage water, and 5 to 10% in river water, respectively. Absolute and relative recoveries of zearalenone over OASIS HLB cartridges were similar to those determined with Supelclean ENVI-18 (*23*).

7.3.2 Mycotoxin emission from experimental fields

Figure 7.2 shows the drainage water runoff (A), concentrations (B, C) as well as the cumulative loads (D, E) of deoxynivalenol and zearalenone in drainage water of the experimental wheat field from mid of June to end of July 2007. Subsurface runoff (Figure 7.2A) was caused by three major rain events before wheat harvest on July 23, and by one rain event thereafter. Deoxynivalenol concentrations in drainage waters ranged from 23 ng/L to 4.9 µg/L (Figure 7.2B) and were roughly two orders of magnitude higher than those observed for zearalenone (max. 35 ng/L) (Figure 7.2C). We suspect two major causes for this pronounced difference: deoxynivalenol usually prevails over zearalenone by about a factor of 20 in terms of average concentrations in wheat (*12*); and estimates of the hydrophobicity of zearalenone (as expressed by its octanol-water partitioning coefficient, K_{ow}) are several orders of magnitude higher than that for deoxynivalenol (*22*). Several metabolites of ZON were routinely analyzed as well (*23*), but could never be detected.

Throughout the investigation, a total of 653 and 3.2 mg/ha deoxynivalenol (Figure 7.2D) and zearalenone (Figure 7.2E), respectively, were emitted via subsurface

runoff. Somewhat smaller amounts were observed for zearalenone during the preceding cultivation periods starting in August 2005 with wheat (0.17 mg/ha), oil radish (0.10 mg/ha), and maize (0.18 mg/ha). These loads (Figure 7.2D, E) correspond to about 1.2% and 0.02% of the total amounts of deoxynivalenol and zearalenone present in the whole wheat plants at the time of harvest (50 and 15 g/ha, respectively). The pre-harvest emission dynamics of deoxynivalenol and zearalenone were markedly different: while about 90% of the emitted deoxynivalenol load was released during one single rain event (July 2-3, Figure 7.2D), zearalenone eluted more continuously over several rain events between July 2 and 23 (Figure 7.2E). Among other factors, the higher K_{ow} of zearalenone might have led to retarded emission and transport relative to deoxynivalenol. A considerable part of the total deoxynivalenol and zearalenone load was released from the field after wheat harvest on July 23. This result can be explained by the fact that only a certain fraction of the total mycotoxin amount (47% of deoxynivalenol, 55% of zearalenone) was present in the wheat kernels, whereas the remainder was distributed between glumes, stems, and leaves, which remained on the field.

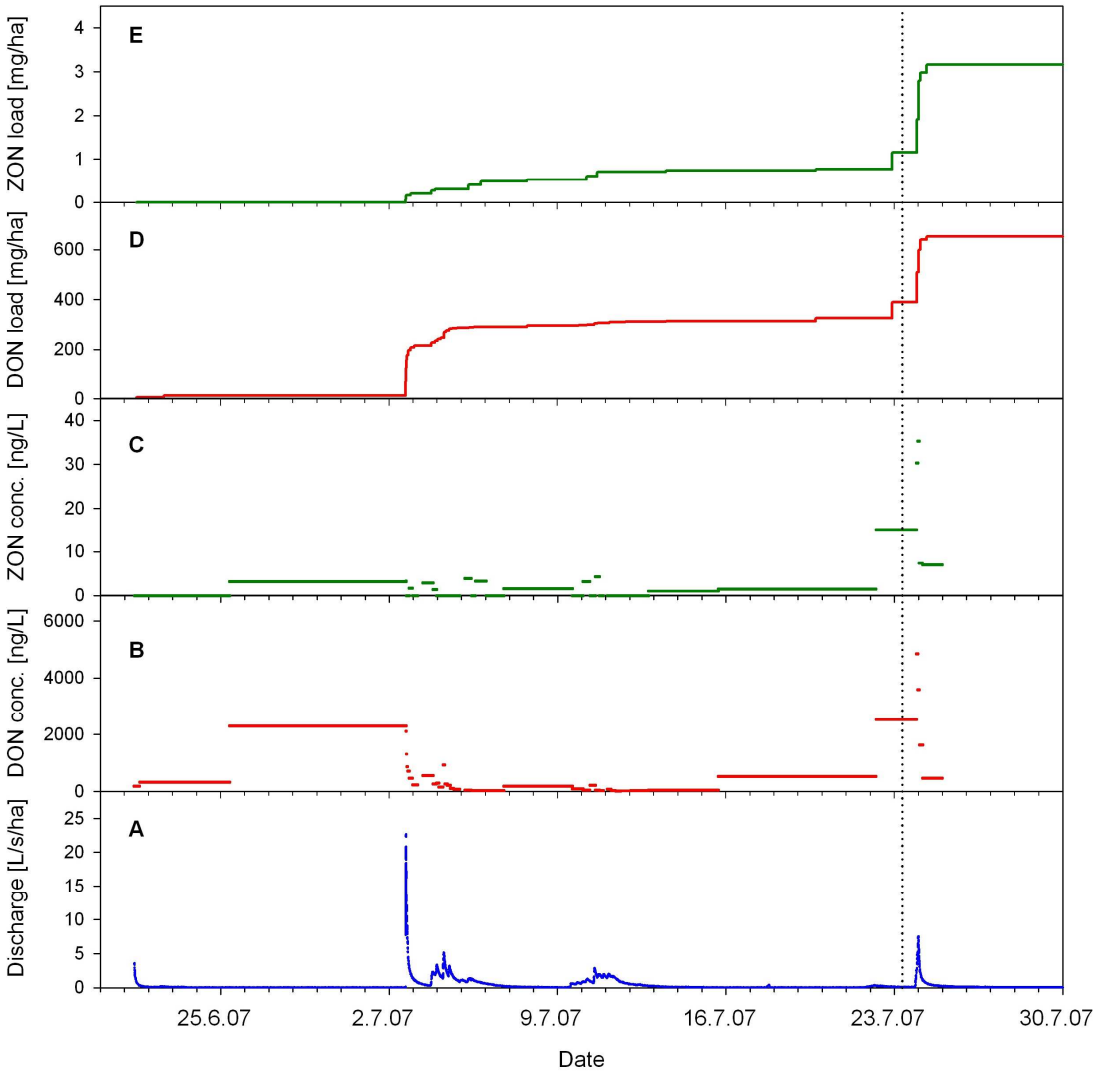


Figure 7.2: Deoxynivalenol and zearalenone in drainage water from a *Fusarium graminearum*-infected experimental winter wheat field. (A) drainage water discharge (L/s/ha). (B) deoxynivalenol concentrations (ng/L). (C) zearalenone concentrations (ng/L). (D) cumulative deoxynivalenol load (mg/ha). (E) cumulative zearalenone load (mg/ha). The dotted vertical line indicates the time of harvest.

7.3.3 Surface water monitoring

During another rain event on August, 8 2007, we traced deoxynivalenol in the recipient water (Chatzenbach) of the drainage water. Concentrations in Chatzenbach were 11, 16, and 19 ng/L in samples taken within 35 min at 400, 1800, and 3900 m, respectively, downstream of the drainage water inflow. No deoxynivalenol was detected in an upstream control sample. This indicates that even a small single contaminated area as our experimental field is sufficient to cause the presence of mycotoxins in surface waters.

In July and August 2007, we detected deoxynivalenol in two major rivers systems of the Canton of Zurich in 60% of all samples. Concentrations ranged from not detected

to 22 ng/L and translated to weekly loads of up to 57 g (Figure 7.3). No deoxynivalenol was detected at Rämismühle, the most upstream sample location of the river Töss. To this point, the river drained only a small wheat area. Further downstream however, the rivers Kempt and Eulach, two tributaries with considerable higher wheat areas in their catchment, brought with them deoxynivalenol in concentrations of up to 8.5 ng/L, corresponding to some 22 g/week (Figure 7.3). Dilution caused the concentrations to largely drop below limits of detection at Freienstein, the river mouth before entrance into river Rhine.

The rivers Aabach and Aa both enter the Greifensee, from which the river Glatt elutes. They contained deoxynivalenol in concentrations as high as 22.0 ng/L, and transported up to 57 g of deoxynivalenol per week (Figure 7.3). In contrast, no deoxynivalenol was quantified at the outlet of Greifensee (Fällanden). This is plausible given the dilution, possible degradation, and the water residence time in the epilimnion of about 150 days. Consequently, the 10.9 ng/L detected during week 29 (44 g/week) in river Glatt at Oberglatt must have largely originated from wheat fields downstream of Greifensee. Only a little more deoxynivalenol was added until the river mouth at Rheinsfelden. Deoxynivalenol was also analysed in samples from rivers such as Aare, Saane, Thur, and Rhine, but amounts were permanently below the quantification or detection limit. This can be rationalized with the much lower wheat area in the catchments of these larger rivers relative to their water discharge. This ratio was 0.1 $km^2/m^3/s$ at Rhine Rekingen, whereas it ranged from 1.0 to 1.8 $km^2/m^3/s$ in the river Töss, and 0.3 to 4.8 $km^2/m^3/s$ in the river Glatt catchment. Corresponding numbers of the two experimental fields were 46 and 384 $km^2/m^3/s$, respectively. Hence, in case of *F. graminearum* infection, deoxynivalenol and zearalenone concentrations in the range of a few ng/L to a few □g/L as emitted in drainage water are roughly 10 to 1000 times diluted in larger surface water bodies.

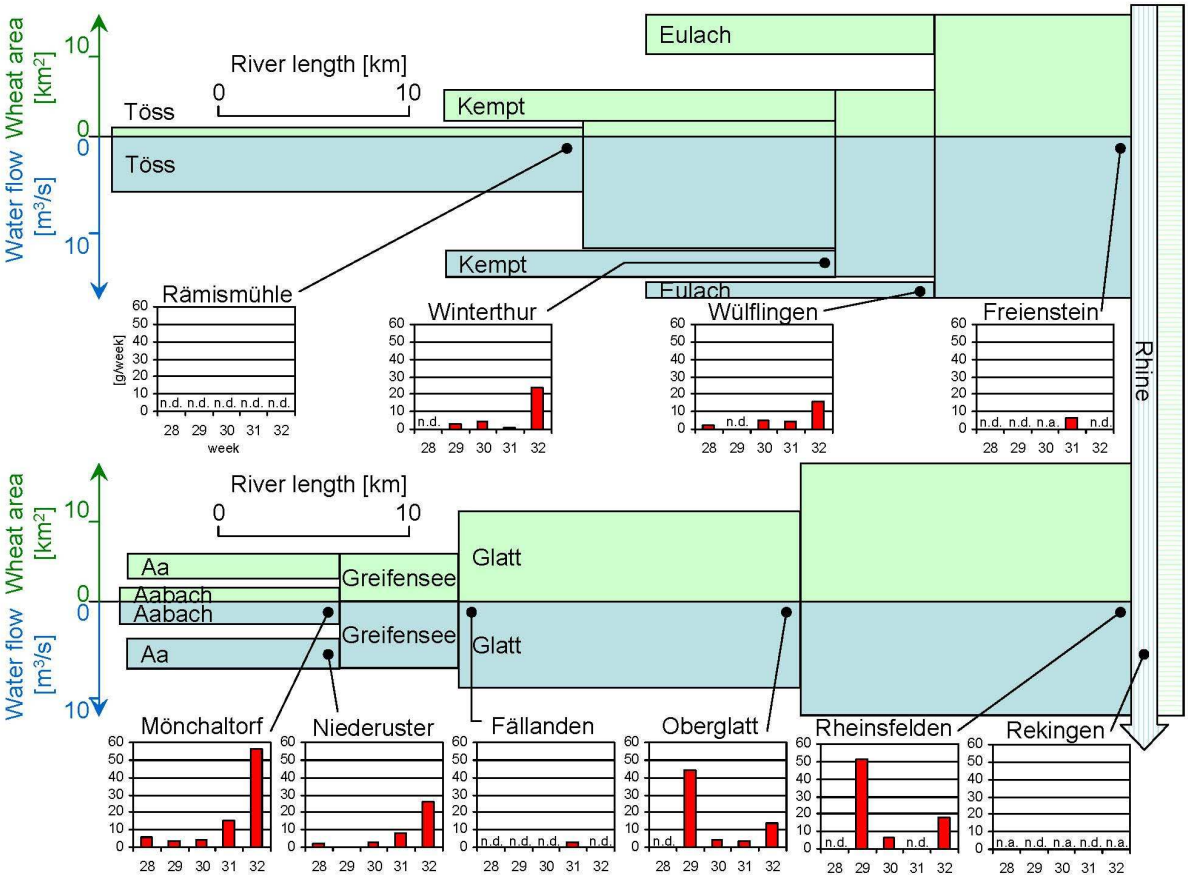


Figure 7.3: Deoxynivalenol loads quantified in the river Töss and river Glatt catchment at the time of winter wheat harvest in 2007. Inserted plots show the temporal development of deoxynivalenol loads (g/week) from July 9 to August 12, corresponding to calendar week 28 to 32. For clarity, axes titles are only given once (Töss, Rämismühle), and are omitted in the other plots. The width of green and blue boxes represent winter wheat area per catchment (km^2), and average water flow Q (m^3/s) as observed during the period of investigation, respectively. Respective numbers of river Rhine at Rekingen (hatched boxes not to scale) are 72 km^2 and 610 m^3/s. n.a.: not analysed. n.d.: not detected.

Note that contamination by deoxynivalenol and zearalenone in the above described manner can only be expected in case of *Fusarium* spp. prevalence, especially *F. graminearum*, *F. culmorum* and *F. crookwellense* (*2*). Weather conditions during anthesis/wheat flowering of 2007 were favourable for *Fusarium* spp. infection, i.e. moist and warm on many sites. Indeed, during the flowering period in June 2007, and in contrast to 2005 and 2006, our deoxynivalenol forecast system FusaProg (*25, 26*) indicated for many Swiss regions mostly suitable weather conditions for *F. graminearum* infection. Accordingly, zearalenone, which was never detected during the preceding two years of monitoring, was detected four times between July 23 and August 5, 2007 (river Aabach at Mönchaltdorf, river Glatt at Oberglatt, and twice in river Eulach at Wülflingen). This result is in line with earlier reports on the occasional occurrence of zearalenone in German and Italian rivers (*16-20*), and renders runoff

from *Fusarium*-infected agricultural fields another, and maybe more plausible source for zearalenone contamination, than cattle excretion.

7.3.4 Possible environmental exposure to other Fusarium mycotoxins

Apart from trichothecenes (e.g., deoxynivalenol) and resorcyclic acid lactones (e.g., zearalenone), a wide range of mycotoxins, such as fumonisins, enniatins, beauvericines, and moniliformin, is produced by a variety of *Fusarium* species on cereals and maize. For a given mycotoxin, the amount produced, and its physical-chemical properties may be used for a first assessment of their environmental emission potential. Concentrations of enniatins in cereals can very well be in the order of mg/kg (*27, 28*), i.e., similar to those of deoxynivalenol. Among all mycotoxins, moniliformin might dominate in runoff and drainage water because it is produced in high amounts (*29*), and because of its anionic, and thus readily water soluble speciation. Overall, we suspect a wider range of mycotoxins to be emitted into the environment in the manner presented here with deoxynivalenol and zearalenone and advocate a systematic surface water screening.

7.3.5 Ecotoxicological relevance

The ecotoxicological consequences of the occasional presence of mycotoxins such as deoxynivalenol and zearalenone in the ng/L to µg/L range in aqueous environments remain to be elucidated. We assume that in most surface waters, zearalenone from agricultural runoff will be diluted to concentrations well below environmental relevance. However, in small water bodies receiving mainly runoff from wheat and maize fields and in case of *F. graminearum* attacks, zearalenone might contribute to the total estrogenicity. As for other mycotoxins, including deoxynivalenol, their ecotoxicological significance is largely unknown, since they are normally considered a risk for food and feed only, and thus do not undergo ecotoxicity testing. It is known however that enniatins and beauvericins (*30*), as well as certain trichothecenes (*31*), exhibit insecticidal effects.

7.3.6 Mycotoxins as micropollutants - comparison with pesticides

To further evaluate the significance of the presence of mycotoxins in the agro-environment, their amounts and concentrations detected in drainage and river water

can be compared with those of pesticides. First, the 50 and 15 g/ha of deoxynivalenol and zearalenone present on a severely contaminated wheat field are, although at the lower end, comparable with application rates of pesticides used: in Switzerland, atrazine is applied up to 1 kg/ha, whereas certain more modern pesticides are used at amounts as low as 50 g/ha. Second, the fraction subject to runoff of 1.2% and 0.02% for deoxynivalenol and zearalenone are well within the range of relative losses of the three commonly applied herbicides atrazine, dimethenamid and metolachlor in small scale catchments draining into river Aa (0.0002% – 1.0%) (*32*). Third, concentrations of deoxynivalenol of up to 20 ng/L as found in rivers of the Canton of Zurich are comparable with those of pesticides. For instance, 50% of all atrazine values (n=653) ranged from not detected to 50 ng/L. Eleven out of 54 analysed pesticides were never detected and six others were only occasionally detected with maximum concentrations <50 ng/L (*33*). Therefore, it seems justified to designate mycotoxins, similar to pesticides or other natural compounds such as cyanotoxins or human hormones, as micropollutants in aquatic systems (*34*).

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Chapter 8: General conclusions

The main goal of this work was to investigate the environmental distribution of zearalenone (ZON). Therefore we first developed analytical methods (Chapter 2 and 3) for ZON quantification in various agro-environmental compartments such as drainage, surface and waste water, soil, manure, plant material and sewage sludge. The figures of merit proved that the developed analytical methods were optimized to quantify ZON at the required levels and with the desired accuracy. In particular, the use of D_6-ZON as internal standard allowed overcoming matrix related problems during sample preparation and ionization of the analyte at the LC-MS interface.

ZON concentrations quantified in maize and wheat from our field site (Chapter 4) were between 0.1 and 16.6 $\mu g/g_{\text{dry weight}}$, which were similar to levels published in numerous works from different countries in Europe and throughout the world. To our knowledge this work showed for the first time the detailed ZON distribution within maize and wheat plants organs. Therewith the quantification of ZON amounts - associated with plant debris - remaining on such fields after harvest was possible. In our field study such ZON amounts were between 6 and 25 g/ha, representing the major input source to the soil compartment (Chapter 6). The considerable variations of these amounts reflected the natural variability caused mainly by variable wheater conditions. ZON input to the soil was additionally influenced by the accessibility of this fraction to be washed off during rain events. Sorption, desorption and probably degradation processes took place concomitantly and prevented an apparent correlation between ZON amounts in the crops and in the soil. As described in chapter 5, sorption was mainly caused by the organic carbon fraction in the soil whereas clay minerals and iron oxides played only a minor role. Dissipation processes such as degradation and sequestration are still poorly studied and it is necessary to investigate this issue in more detail for predicting ZON concentrations in soils.

Other possible ZON input sources to soil such as direct animal excretion, manure, sewage sludge and waste water were discussed in chapter 3 and 6. ZON input via animal excretion and manure were calculated to be in the same order as those washed off from *Fusarium* infested maize and wheat fields. In sewage sludge we quantified ZON in only two cases. Therefore, and because sewage sludge was recently banned as fertilizer in Switzerland, this input source is not relevant. However, this use is common practice in other countries. In contrast to earlier publications, we did not find ZON in the investigated waste water effluent samples.

Even though not investigated within the accomplished project, the application of compost and digestate used as soil improver and fertilizer should be kept in mind as another potential input source of ZON to soil, because ZON contaminated wheat straw and residues from cereal processing may be used as input material.

ZON desorption from contaminated soils seems to be negligible, as suggested by the data on emission via drainage water (Chapter 2 and 4). This process was rather driven by preferential flow, which is nicely shown by the correlation of ZON concentrations in the drainage water and the amount of discharge. ZON concentrations in the drainage water were mainly in the very low ng/l range throughout the investigated period of largely two years. However, occasional peak concentrations may have reached environmentally critical levels.

Our data clearly showed that soil was the primary recipient of ZON emitted from *Fusarium* infested wheat and maize fields. Unfortunately, our knowledge about the consequences of endocrine disruptors in soil is still very limited. ZON concentrations in the drainage water were similar to 17β-estradiol levels found in surface waters. Due to the large dilution of drainage water in surface waters, ZON concentrations will usually drop below detection limit and probably environmentally critical levels, although little is known about ecotoxicological consequences of pulsed exposures (Chapter 4). In several hundred investigated surface water samples taken throughout the midlands of Switzerland, ZON was detected in four cases only (Chapter 6). Still, in small creeks mainly fed by agricultural runoff, ZON might contribute substantially to the total estrogenicity of such water in case of *Fusarium* occurrence. As described in chapter 7, ZON was not the only mycotoxin present in the drainage water from *Fusarium* infested crop fields. In particular, the well known deoxynivalenol (DON) was quantified in concentrations much higher than these of ZON. As a consequence, DON was also found in large surface water bodies of the Canton of Zurich, such as the Glatt and Toess, in concentrations similar to those of modern pesticides. Therefore, we suggest extending the quest for other mycotoxins acting as aquatic micropollutants.

Appendix: Supporting information of Chapter 4

Supporting information

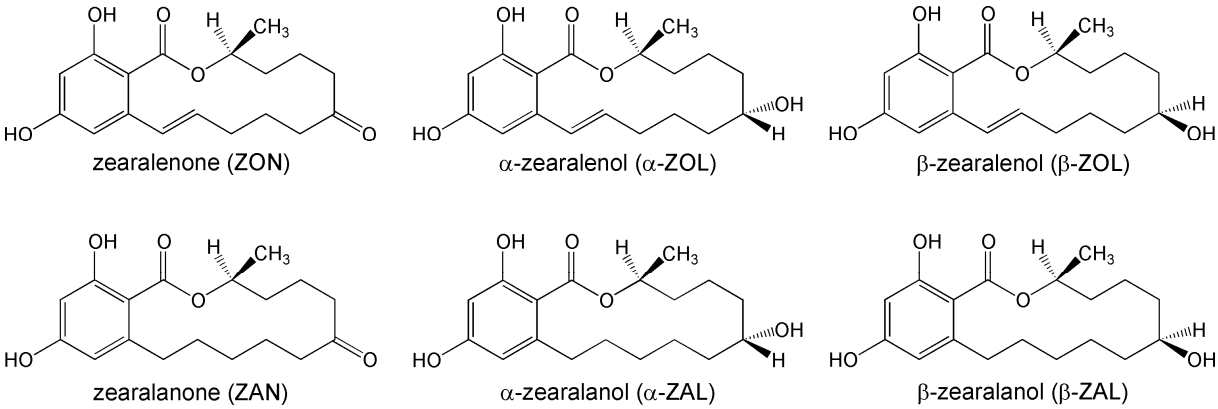

Figure S1: Chemical structures, names and abbreviations of all six resorcyclic acid lactones.

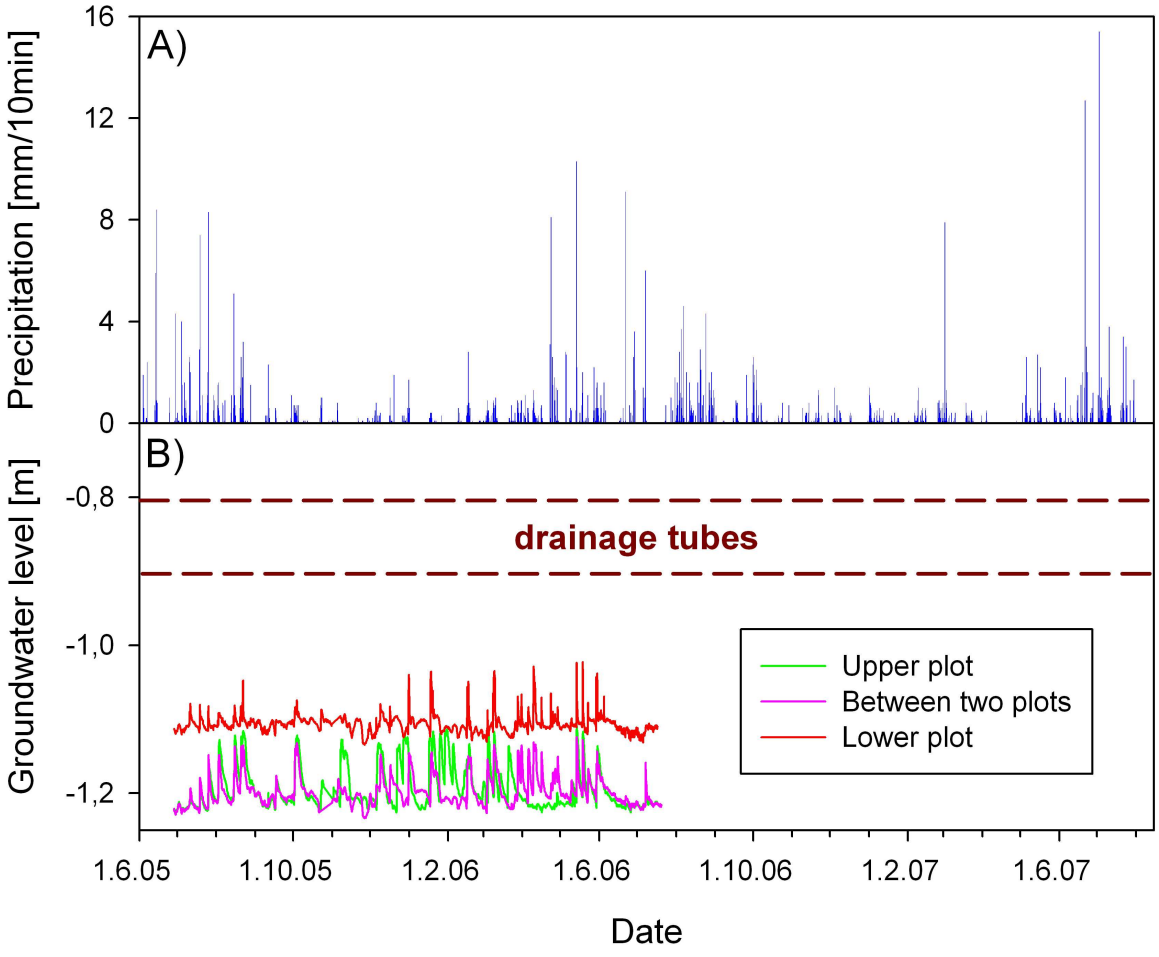

Figure S2: Precipitation and groundwater level. A) Precipitation in mm/10min collected from the meteorological station close to the field site, B) groundwater levels measured on the upper plot (green line), between the two plots (pink line) and on the lower plot (red line). Brown dashed lines indicate the depth where the drainage tubes were situated.

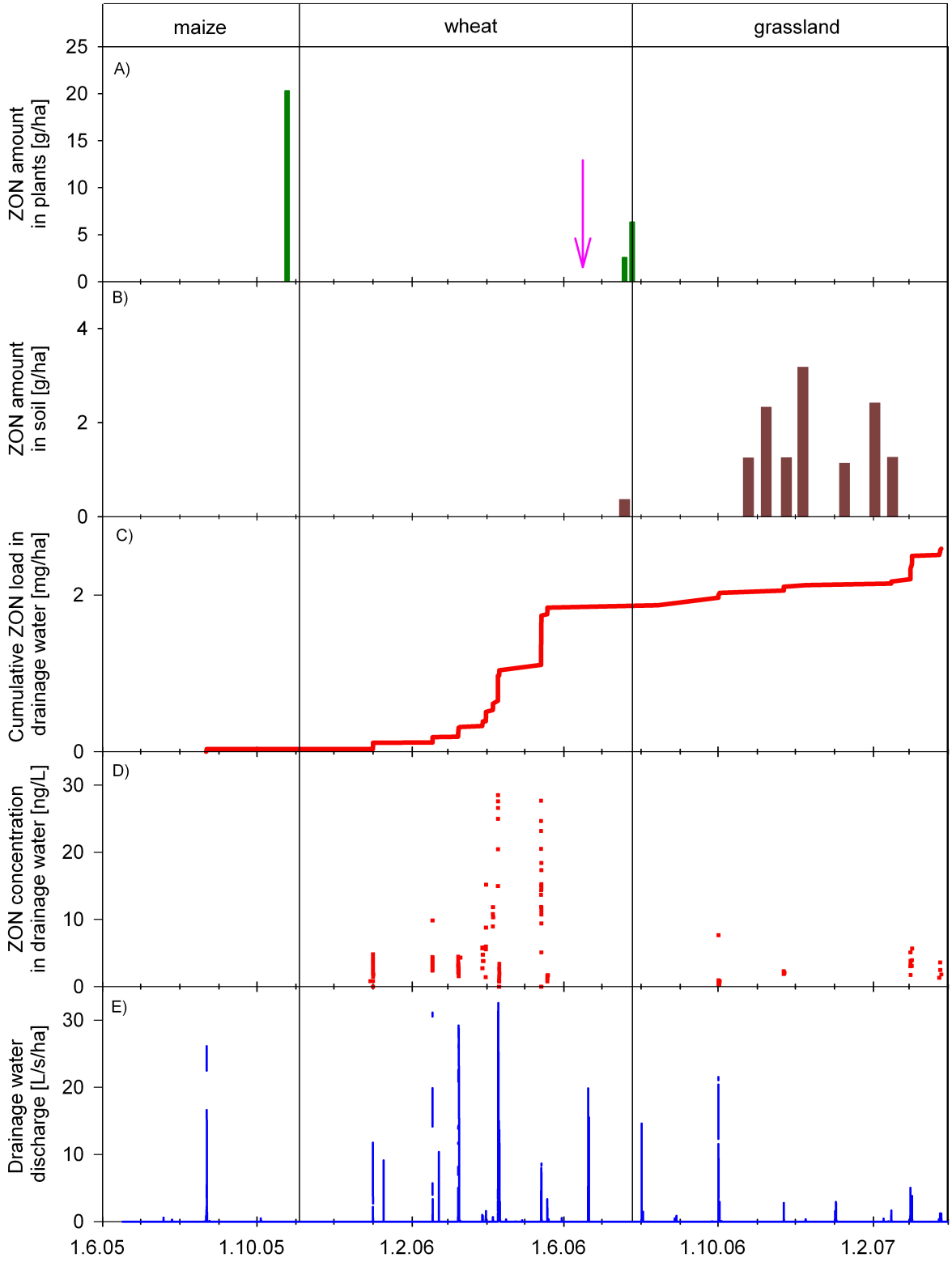

Figure S3: Occurrence of zearalenone (ZON) on *Fusarium graminearum* infested wheat and maize fields over a two year crop rotation on the lower plot. A) ZON amounts in the cultivated plants, B) ZON amounts in the topsoil (0-10cm), C) ZON loads emitted via drainage water, D) ZON concentrations in the drainage water and E) drainage water discharge. Pink arrows indicate the time of *Fusarium* infestation of the wheat field.

Table S1: Field soil parameters [a]

Soil horizon	Sampling depth [cm]	pH ($CaCl_2$)	C_{org} [%]	Humus [b] [%]	Clay [%]	Silt [%]	Sand [%]
Ahp	0 - 20	6.8	2.0	3.4	30.6	39.4	30.0
Bg	20 - 35	7.0	2.6	4.5	44.2	28.9	26.9
Bgg	35 - 50	7.0	0.9	1.5	30.0	38.2	31.8
BCgg	50 - 70	7.7	0.2	0.4	18.2	49.6	32.2
Cgg	70 - 90	7.8	0.1	0.1	13.2	66.3	20.5

[a] Data collected at Agroscope Reckenholz-Tänikon ART, Reckenholz, 8046 Zürich, CH
[b] Calculated: C_{org} multiplied by a factor of 1.72

Table S2: Drainage water discharge corresponding to drainage water sample

Period	Discharge per sample
Upper plot	[L]
June 6, 2005 - Aug 20, 2005	250
Aug 20, 2005 - Sept 2, 2005	500
Sept 2, 2005 - Aug 7, 2006	250
Aug 7, 2006 - Nov 21, 2006	5000
Nov 21, 2006 - Nov 23, 2006	1700
Nov 23, 2006 - Dec 9, 2006	5000
Dec 9, 2006 - May 17, 2007	20000
May 17, 2007 - Aug 15, 2007	5000
Lower plot	[L]
Aug 1, 2005 - Mar 9, 2006	250
Mar 9, 2006 - May 14, 2006	500
May 14, 2006 - Dec 9, 2006	2500
Dec 9, 2006 - Mar 26, 2007	2000

Curriculum Vitae

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08/2004 – 04/2008	Ph.D thesis at Agroscope Reckenholz-Tänikon ART
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