JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Fusarium Mycotoxins: Overlooked Aquatic Micropollutants?

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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 liquid chromatography−mass spectrometry (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 *Fusarium 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, because 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.

KEYWORDS: Trichothecenes; resorcyclic acid lactones; runoff; endocrine disruption; natural estrogens; *Fusarium* head blight; $^{13}C_{15}$-deoxynivalenol; isotope-labeled internal standard; analysis

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 2.7 billion U.S. dollars in U.S. wheat and barley production between 1998 and 2000 (*5*). The most prevalent mycotoxins produced by *Fusarium* spp. include deoxynivalenol and zearalenone (*1, 2*) (**Figure 1**). Zearalenone, in particular, is a potent natural estrogen (*6–9*).

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 0.2−1, and 6−11 days, respectively. Several recent papers reported the occasional occurrence of resorcyclic acid lactones in wastewater 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 have been banned in the European Union (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 *Fusarium graminearum*-infested agricultural fields. For the current study, we have extended our ongoing investigations on the environmental fate and behavior 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 nanogram per liter concentration range, using

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10.1021/jf073082k CCC: $40.75
Published on Web 01/16/2008

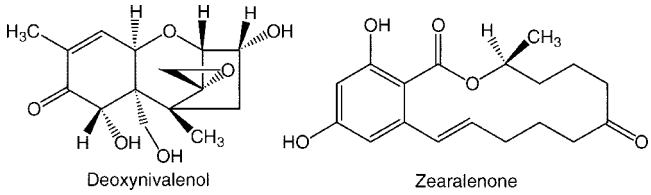

Figure 1. Structures of deoxynivalenol and zearalenone.

$^{13}C_{15}$-deoxynivalenol as an internal standard. Deoxynivalenol and zearalenone were then quantified concomitantly in drainage water and in Swiss river water systems around the period of wheat harvest.

MATERIALS AND METHODS

Deoxynivalenol and Zearalenone Analysis. Deoxynivalenol and zearalenone were solid-phase-extracted from aqueous samples, followed by separation and detection with liquid chromatography−tandem mass spectrometry (LC−MS/MS). 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 fiber filters, 1.2 μm) 1 L water samples, prior to extraction of deoxynivalenol and zearalenone with OASIS HLB (6 cm^3, 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 5−10 mL/min. The cartridges were subsequently washed with 5 mL of Milli-Q water and dried by vacuum. Finally, the analytes were eluted with 4 mL of ethyl acetate into conical microreaction 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 °C and analyzed 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 × 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; and 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 5 mM 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, −600 V; drying gas (N_2, 99.5%), 225 °C and 1.23 bar; nebulizing gas (compressed air), 3.34 bar; vaporizer gas (N_2, 99.5%), 310 °C and 0.90 bar; housing temperature, 50 °C. 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 2.67×10^{-6} bar, and the detector voltage was set to 1800 V. 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 (version 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*).

Experimental Fields. Two adjacent but separately drained 0.2 ha 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 of sample/1000 L of water, pooled to yield 1 L samples). This type of sampling allows us to quantify 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 multiyear crop rotation, the following conditions favorable 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 analyzed 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.

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 on the basis of 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 analyzed for deoxynivalenol from July 9 to August 12, 2007 (calendar weeks 28−32, 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 28−31, four samples per station within this period). Deoxynivalenol loads were calculated from the quantified concentrations and the averaged daily water flows, which were obtained from the AWEL and NADUF sampling station protocols.

RESULTS AND DISCUSSIONS

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 nanograms per liter 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 and 50 000 pg. Ion suppression for deoxynivalenol was 18 and 14%; absolute method recoveries were 85 and 87%; and relative recoveries were 91 and 101% in drainage water and river water, respectively. Method detection limits derived from 3 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 5−12% in drainage water and 5−10% in river water, respectively. Absolute and relative recoveries of zearalenone over OASIS HLB cartridges were similar to those determined with Supelclean ENVI-18 (*23*).

Mycotoxin Emission from Experimental Fields. Figure 2 shows the drainage water runoff (**A**), concentrations (**B** and **C**), as well as the cumulative loads (**D** and **E**) of deoxynivalenol

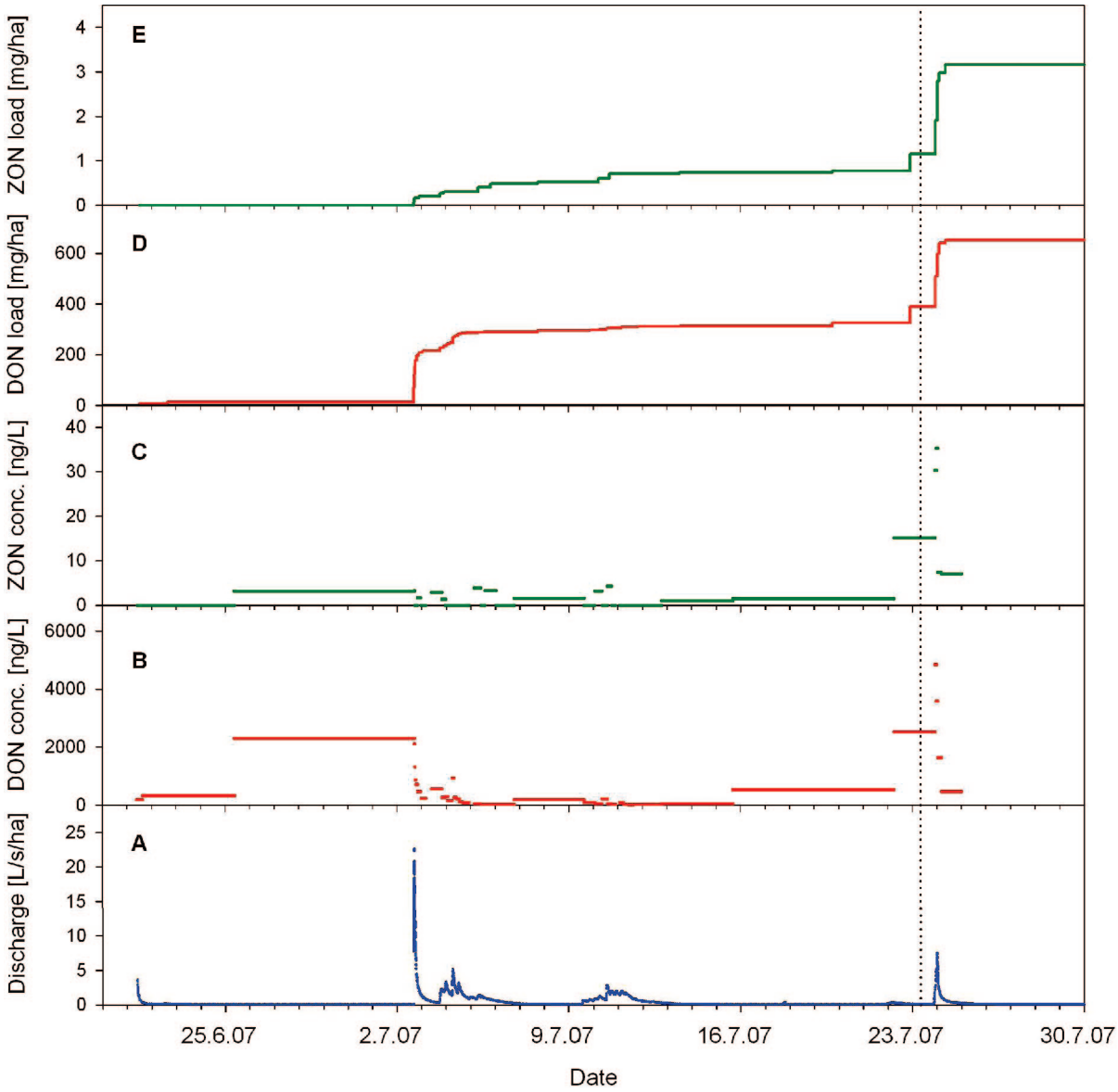

Figure 2. Deoxynivalenol and zearalenone in drainage water from a *F. graminearum*-infected experimental winter wheat field. (**A**) Drainage water discharge (L s^{-1} ha^{-1}). (**B**) Deoxynivalenol concentrations (ng/L). (**C**) Zearalenone concentrations (ng/L). (**D**) Cumulative deoxynivalenol load (mg/ha). (**E**) Cumulative zearalenone load (mg/ha). The dotted vertical line indicates the time of harvest.

and zearalenone in drainage water of the experimental wheat field from the middle of June to the end of July 2007. Subsurface runoff (**Figure 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 2B**) and were roughly 2 orders of magnitude higher than those observed for zearalenone (maximum of 35 ng/L) (**Figure 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 2D**) and zearalenone (**Figure 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 (parts **D** and **E** of **Figure 2**) 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 preharvest 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 2D**), zearalenone eluted more continuously over several rain events between July 2 and 23 (**Figure 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 and 55% of zearalenone) was present in the wheat kernels, whereas the remainder was distributed between glumes, stems, and leaves, which remained on the field.

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 Chatzen-

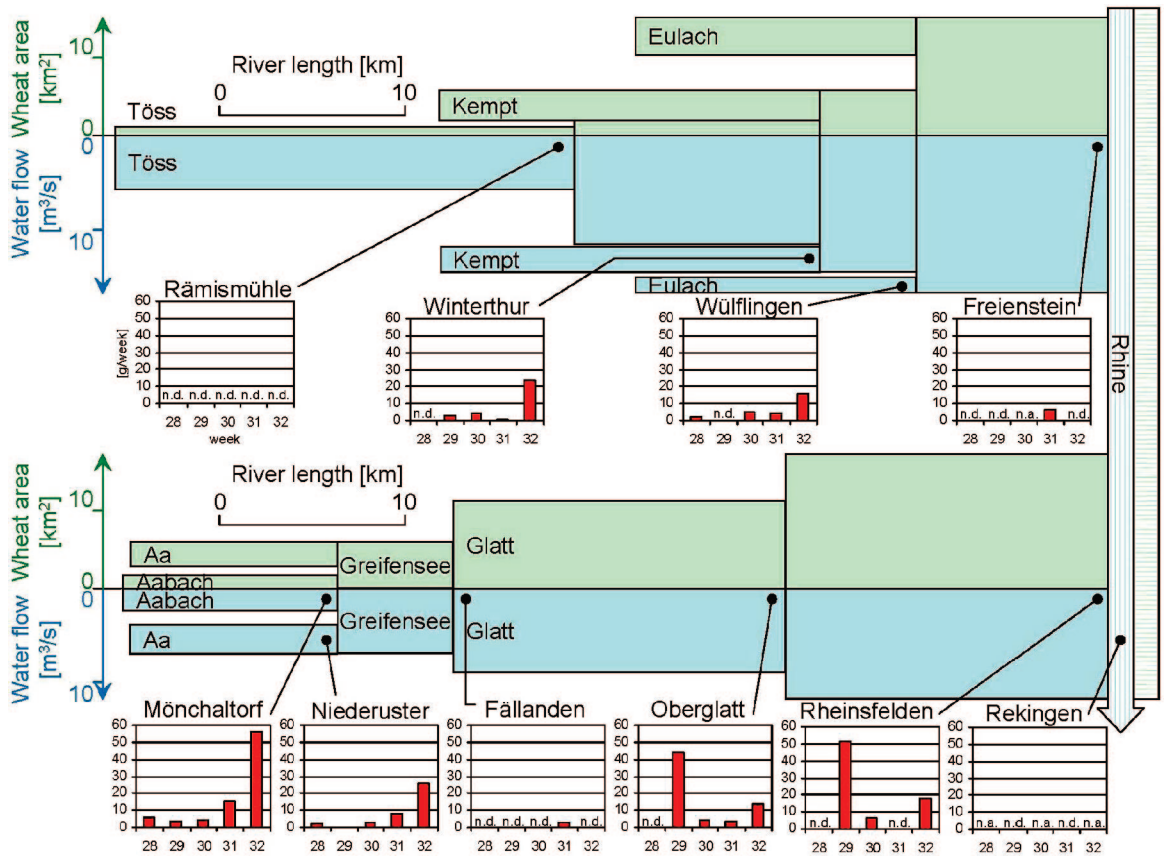

Figure 3. Deoxynivalenol loads quantified in the rivers Töss and Glatt catchment at the time of winter wheat harvest in 2007. Inserted plots show the temporal development of deoxynivalenol loads (g/week) from July 9 to August 12, corresponding to calendar week 28−32. For clarity, axes titles are only given once (Töss and 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.

bach 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 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 3**). Dilution caused the concentrations to largely drop below limits of detection at Freienstein, the river mouth before the 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 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 analyzed 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 $km^2\ m^{-3}\ s^{-1}$ at Rhine Rekingen, whereas it ranged from 1.0 to 1.8 $km^2\ m^{-3}\ s^{-1}$ in the river Töss and from 0.3 to 4.8 $km^2\ m^{-3}\ s^{-1}$ in the river Glatt catchment. Corresponding numbers of the two experimental fields were 46 and 384 $km^2\ m^{-3}\ s^{-1}$, respectively. Hence, in case of *F. graminearum* infection, deoxynivalenol and zearalenone concentrations in the range of a few nanograms per liter to a few micrograms per liter as emitted in drainage water are roughly 10−1000 times diluted in larger surface water bodies.

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 favorable 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 2 years of monitoring, was detected 4 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 as another and maybe more plausible source for zearalenone contamination than cattle excretion.

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 milligrams per kilograms (*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.

Ecotoxicological Relevance. The ecotoxicological consequences of the occasional presence of mycotoxins, such as deoxynivalenol and zearalenone, in the nanogram per liter to microgram per liter 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, because 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.

Mycotoxins as Micropollutants—Comparison to 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 to 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 to the 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 to those of pesticides. For instance, 50% of all atrazine values ($n = 653$) ranged from not detected to 50 ng/L. A total of 11 of 54 analyzed pesticides were never detected, and 6 others were only occasionally detected with maximum concentrations of <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*).

ACKNOWLEDGMENT

We thank H. J. Bachmann for reviewing the manuscript. We gratefully acknowledge W. Meier (AWEL) and B. Luder (NADUF) for providing surface-water samples from different sampling stations throughout Switzerland. Furthermore, we are thankful to the field staff at ART for their excellent support at the field site.

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Received for review October 19, 2007. Revised manuscript received December 10, 2007. Accepted December 10, 2007. This work is part of the project "Environmental Exposure to Estrogenic Mycotoxins" within the National Research Programme NRP50 "Endocrine Disruptors: Relevance to Humans, Animals and Ecosystems" financed by the Swiss National Science Foundation. We also thank G. Karlaganis and S. Müller (Swiss Department of Environment, BAFU) for financial support.

JF073082K