Environmental Sciences Europe
a SpringerOpen Journal

RESEARCH Open Access

Estrogenic activity in drainage water: a field study on a Swiss cattle pasture

Andreas Schoenborn[1*], Petra Kunz[2] and Margie Koster[3]

Abstract

Background: Dairy cow manure applied to pastures is a significant potential source of estrogenic contamination in nearby streams. One possible pathway is through infiltration via preferential flow to drainage pipes, particularly after heavy rainfall events. In a period of 73 days in the spring of 2010, a drainage catchment in a cattle pasture in the Swiss lowlands was closely monitored.
Manure was applied three times during the study, and part of the catchment was also subjected to grazing. During five field campaigns, water samples from two sampling locations were taken for 4–24 h in consecutive sampling intervals. 17β-estradiol equivalents (EEQ) were determined with the yeast estrogen screen (YES) and the ER-CALUX assay. Some water chemistry parameters, pH, conductivity, oxygen content and soil moisture tension were also monitored.

Results: Washout of estrogenic activity was highest during or right after heavy rainfall events, shortly after manure spreading, when peak values of >10 ng/l EEQ were found in several samples. However, in two field campaigns, high EEQ values were also found 14 and 28 days, after the last manure application, in one case during a dry weather period. This indicates that estrogenic compounds are more stable in natural soils than what is expected from data gathered in lab studies.

Conclusions: Streams in agricultural areas with a high proportion of drained land may be subject to numerous peaks of EEQ during the course of the year. This may have a negative effect on aquatic organisms, namely fish embryos, living in these streams.

Keywords: Drainage water; Manure; Dairy cattle; estrogenic activity; EEQ; YES; ER-CALUX; Aquatic organisms

Background

Treated wastewater is a potential source of estrogenic activity of anthropogenic origin in natural water bodies. estrogenic activity in wastewater has been linked to sexual changes in fish [1] and is suspected to be "a major causal factor in the evolution of intersexuality" in roach [2]. Following these findings, estrogens in treated wastewater were closely examined in a number of countries in the last 10–15 years, e.g. in Britain [2], The Netherlands [3], Denmark [4] and Switzerland [5, 6].

The role of agriculture as source of estrogenic activity for natural water bodies has received much less attention. A "normalised cow" excretes two orders of magnitude more and a "normalised pig" one order of magnitude more steroid estrogens than a "normalised human" [7]. A conservative estimate for Switzerland shows that the total annual estrogen load released onto the environment from livestock exceeds that excreted by humans by at least a factor 5 (Table 1). Johnson et al. calculated the same factor 5 for the UK. In a review on sex hormones originating from livestock, Lange et al. [8] concluded that "discussion on environmental endocrine disrupters has to be extended by this important aspect" even though they did not find causal links in literature to "any known severe adverse effect on wildlife or human endocrine system".

A number of studies demonstrate the presence of farm-animal-derived steroid hormones in manure and wastewater from dairy farms [9–11]. Manure is usually spread on soil surfaces or (more recently) is injected. With the exception of karst areas [12] it is still largely unclear to what extent these hormones can reach nearby

* Correspondence: andreas.schoenborn@zhaw.ch
[1]Institute of Natural Resource Sciences, Zurich University of Applied Sciences, P.O. Box CH-8200, Waedenswil, Switzerland
Full list of author information is available at the end of the article

Table 1 Estimated annual oestrogen load in Switzerland (2007) from excreta of humans and three common livestock animals

Species	Excretion of oestrogens (mg/individual/year)	Number of individuals	Annual oestrogen load (kg/year)	Share (%)
Cows	110[c]	708,340[a]	77.9	43
Pigs	43[c]	1,573,090[a]	67.6	37
Sheep	8.4[c]	443,584[a]	3.7	2
Humans	4.38[d]	7,593,500[b]	33.3	18

[a]From Bundesamt für Statistik [36]
[b]From Bundesamt für Statistik [47]
[c]After [25] (cycling females)
[d]Calculated from estimated average excretion of 12 μg/person/day (sum of E1, E2 and E3, pregnant women excluded), after Table two of [48]

water bodies. Sorption/desorption studies showed a rapid degradation and high sorption of estrogens in soils (several studies, as cited in [13]). In one of the few field studies (on grassland soils treated with cattle and sheep manure), Lucas and Jones [14] showed that estrone (E1) and 17ß-estradiol (E2) are "not persistent in agricultural soils" and calculated a half-life from 5–25 days for these two estrogens.

However, individual soil conditions can modify the persistence of estrogens. The presence of sheep urine enhances and prolongs the amount of estrogen leaching from soil [15]. The association with manure-borne dissolved organic carbon (DOC) reduces the bioavailability of estrogens and increases their persistence [16]. Anaerobic conditions slow down the degradation of some estrogens [17]. This coincides with the observation that E2 "was widespread, persisted much longer, and was more mobile than previously determined" in soils of a pig farm in North Dakota, United States of America [18].

One pathway from soil to water is through preferential flow channels and drainage pipes (also called "tile drains" or "mole drains"). For the monitoring of this pathway, the sampling procedure seems to be crucial. Based on grab samples taken in creeks and from "tile drains" on four different dates, [19] found no enhanced estrogen concentrations in surface water collected upstream and downstream of a large confinement dairy operation in the mid-western United States. In contrast to that, a Danish one-year study on two field sites with "structured, loamy soil" [13] found E1 and E2 in the drainage pipes within 14–30 days after application of pig manure slurry as well as continued leaching in high concentrations after 3 months. They sampled drainage water flow-proportionally for approximately 1 day, following the onset of "typical" storm events. Matthiessen et al. [20] used "POCIS" passive samplers to monitor 10 streams in England and Wales, from November 2005 to January 2006. Their study sites lay upstream and downstream of intensive livestock farms and were chosen due to a high predicted steroid load. estrogenic activities were higher in 50 % and steroid concentrations were higher in 60 % of the downstream sites. However, estrogenic activity could not solely be attributed to E1 and E2.

The aim of the field study presented here was to assess the role of dairy cow manure as a source of estrogenic activity in drainage water of a cattle pasture in the Swiss lowlands. Assuming a short half-life of estrogens in soil and a good sorption capacity of the local soil, we hypothesised that peaks of estrogenic activity in drainage water should be highest during or right after heavy rainfall events, ideally shortly after manure spreading. The experimental setting was designed to catch these peaks.

Results and discussion

General conditions at the Guettingen field site

Characterisation of the soil

Three soil horizons were characterised regarding their organic carbon content, volume of macropores and porosity (Table 2). Grain size analyses indicated loam to sandy loam with a low saturated hydraulic conductivity K_{sat} of 1.52×10^{-6} (equivalent to a pK_{sat} of 6.2) (C. Boesiger, ZHAW Bachelor's thesis 2010, unpublished). The infiltration experiment revealed traces of blue stain as deep as 80 cm underneath the surface, demonstrating the importance of preferential flow paths in this soil (C. Boesiger, ZHAW Bachelor's thesis 2010, unpublished).

Manure application and grazing periods

Pastures "West" and "East" (Fig. 1) were fertilised with manure on March 24, 2010, followed by a grazing period (Fig. 2). Shortly before the first field campaign (FC1), on April 21 and 28, two doses of manure were applied to pasture "West", while pasture "East" was grazed until May 12, 2010 (the density of the animals on the pastures was not monitored). During the following cool and wet period, the grass grew slowly and harvesting grass was not possible to avoid soil compaction. Grass was cut after a few warm days on June 9, 2010. On June 15, 2010, shortly before FC4, manure was spread on pastures "West" and "East".

Precipitation and outflow

Precipitation in April, May and June 2010 was 25.6 mm (FC1), 128.3 mm (FC2 and FC3) and 111.8 mm (FC4 and FC5), respectively. April 2010 was drier and warmer, while May and the beginning of June were wetter and cooler than the long-term average of 1976–1990 (source:

Table 2 Soil characteristics at the Guettingen field site

Soil horizon[a]	Depth (cm)	Organic carbon content (%)	Macropore volume (%)	Porosity (%)
Ah	0–17	3.26 ± 0.16	11	48
Bg	17–37	1.497 ± 0.07	10	38
BC	>37	0.269 ± 0.01	11	34

[a]Classification according to Swiss soil taxonomy [49]

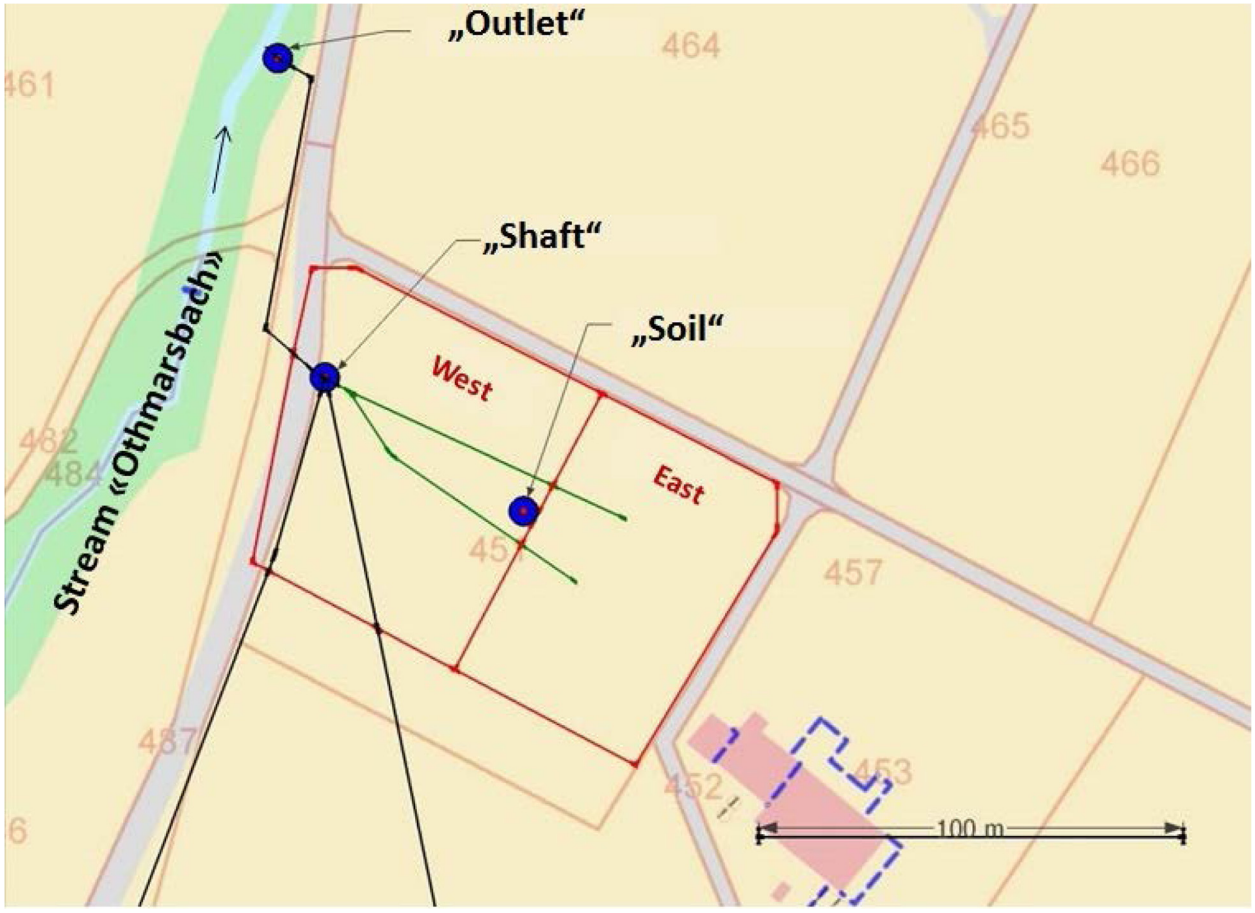

Fig. 1 Sketch of sampling location in Guettingen, Canton of Thurgau, Switzerland, showing the three sampling sites "Shaft", "Outlet" and "Soil", the drainage pipes (*green lines*) collecting at "Shaft" and the pastures "West" and "East". Source of GIS map: Gemeinde Guettingen

MeteoSwiss). FC4 (June 17–18) coincided with heavy rainfall (45.1 mm = 40.3 % of the total precipitation in June 2010).

The regular drainage outflow at "Shaft" lay between 0.05 and 0.1 l/s and exceeded this value only after rainfall events that were not absorbed by the soil. The wet weather conditions from end of April until mid-May led to rapid and transient peak outflow for a few hours following rainfall. After May 13, the general outflow increased moderately, most likely due to the increased groundwater level. Only prolonged rainfall led to persistently higher outflow at "Shaft". Figure 2 gives an overview on precipitation, outflow at "Shaft", pasture management, as well as the dates of the FC manure application and grass cutting.

Chemical and physical characteristics of drainage water

Water quality of the drainage water at "Shaft" (Table 3) reflected the agricultural influence, the geology of the catchment and the weather situation. Average nutrient concentrations (PO_4-P, NH_4-N) at times exceeded the limits set for treated wastewater by Swiss regulations. PO_4-P median concentration was 4.6 times higher and NH_4-N median concentration was 10 times higher than the mean level measured in 2009 during regular monitoring in the stream Hornbach, to which the Othmarsbach contributes. NO_3-N median concentration lay within the range of values found in literature for drains of grassland and pastures [21]. Chloride median concentration lay four to eight times above the natural background concentration of 2–4 mg/l in Switzerland [22].

Under dry weather (base flow) conditions, both hardness level and electrical conductivity were high, and oxygen saturation was mostly close to 100 %, indicating low dissolved organic matter. This shows that base flow consisted of drained groundwater from uphill. Under peak flow conditions, conductivity, in coincidence with rainfall, dropped sometimes sharply within minutes. Sudden rapid drops of oxygen saturation were also observed and coincided with visible leaching of organic material from manure into the drainage pipe. Thus, peak flow consisted of rainwater and sometimes of leached manure.

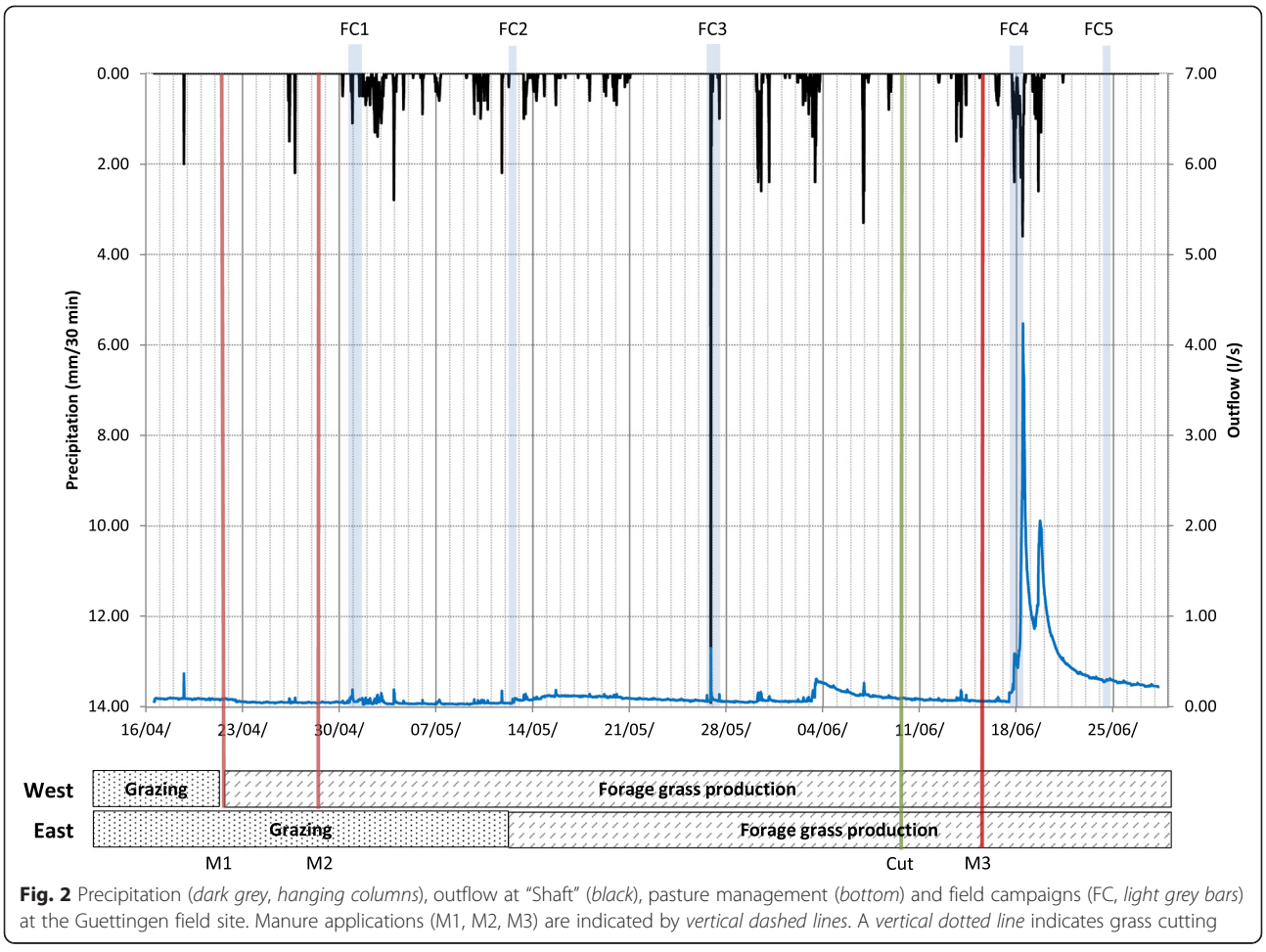

Fig. 2 Precipitation (*dark grey*, *hanging columns*), outflow at "Shaft" (*black*), pasture management (*bottom*) and field campaigns (FC, *light grey bars*) at the Guettingen field site. Manure applications (M1, M2, M3) are indicated by *vertical dashed lines*. A *vertical dotted line* indicates grass cutting

Soil moisture tension

Rainfall and soil moisture tensions (SMT) at 12.5 and 25 cm depth are summarised in Fig. 3. The sensors at 50 cm depth did not work reliably and these data were therefore excluded.

The Ah-horizon (12.5 cm) reached a SMT of >900 hPa (very dry) three times within the field period. After rainfall, soil water tension dropped (sometimes sharply) depending on the amount of rain. The largest drop of SMT was during FC3 (from 510 to 105 hPa within 24 h). In the rainy period between May 1 and May 22, SMT of the Ah-horizon was mostly below 100 hPa (saturated).

In the Bg-horizon (25 cm), the SMT was usually lower than in the Ah-horizon. One of the three exceptions was

Table 3 Results of the water chemistry and physical parameters at "Shaft": measurements between April 16 and June 28, 2010

Parameter	Median	Min	Max	Values below range[c]	Values above range[c]	Hornbach 2009
PO_4-P (mg/l), $n = 21$	0.23	0.06	0.91	3	8	0.05
NO_3-N (mg/l), $n = 21$	8.28	5.93	10.6	0	0	5.15
NH_4-N (mg/l), $n = 21$	0.2	0.02	0.35	9	1	0.02
Chloride (mg/l), $n = 19$	15.9	7.42	23.5	0	0	14.65
Hardness (°dH), $n = 3$	18.5	14.5	18.8	0	11	n.a.
Electrical conductivity (µS/cm), $n = 16$	692[b]	595[b]	786[b]	–	–	611
Oxygen (mg/l), $n = 4498$[a]	9.10[a]	6.12[a]	10.21[a]	–	–	10.55
pH, $n = 16$[b]	7.76	7.01	8.13	0	0	8.2

n.a. no data available
[a]Measurement with Troll 9500
[b]Measurement with Hach HQ40
[c]Measurement ranges: PO_4-P 0.05–1.5 mg/l, NO_3-N 0.23–13.5 mg/l, NH_4-N 0.015–2.0 mg/l, Cl 1–1000 mg/l, hardness 1–20 °dH, electrical conductivity 0.01µS/cm–200 ms/cm

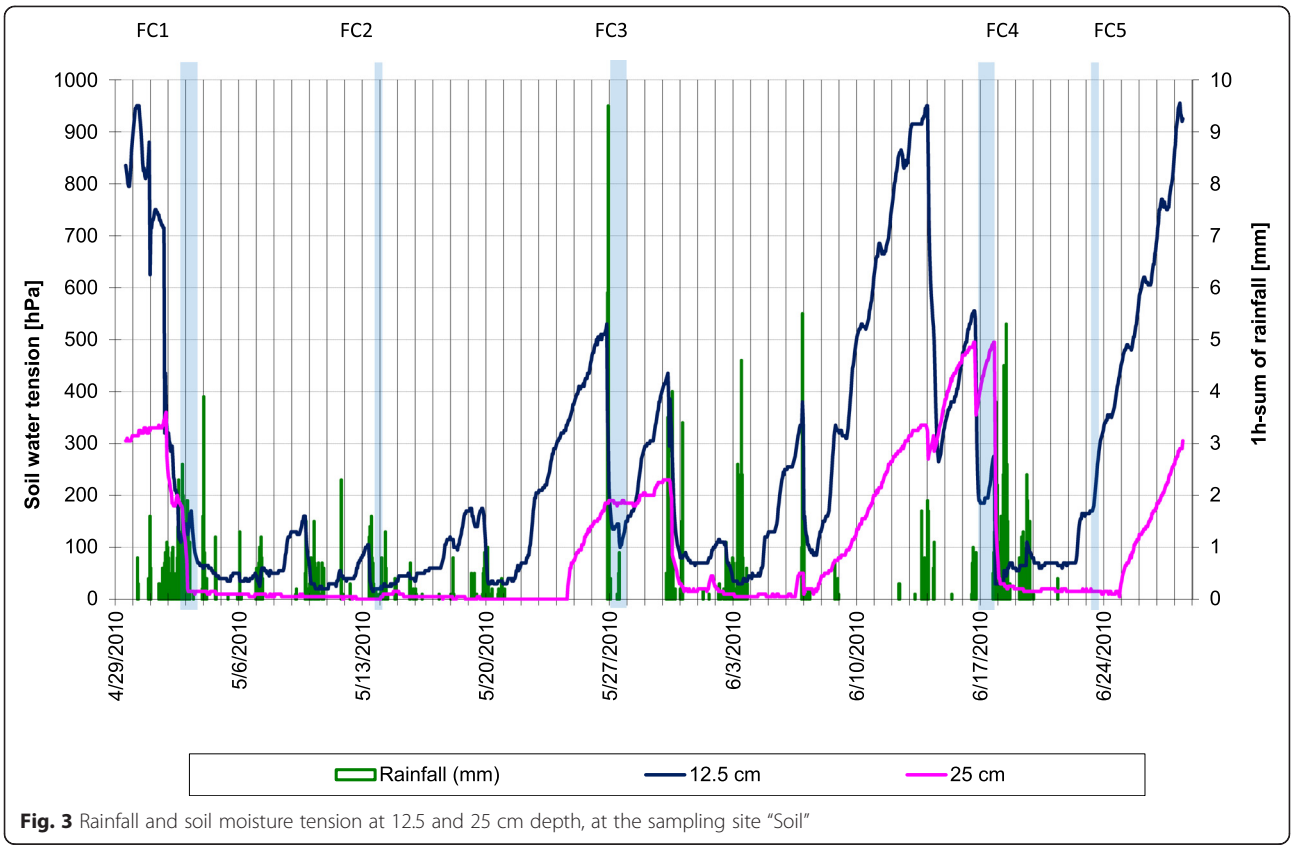

Fig. 3 Rainfall and soil moisture tension at 12.5 and 25 cm depth, at the sampling site "Soil"

during FC3 (May 27–28), when the SMT of the Ah-horizon dropped below the SMT in the Bg-horizon, due to the short but intense rainfall event of FC3. In the rainy period between May 3 and May 24, the Bg-horizon was completely saturated, with an SMT <15 hPa from May 3 to May 24.

estrogenic activity of manure and drainage water samples

estrogenic activity and bioassays

Receptor-based estrogen assays examine the sum of all estrogenic activity in a sample by measuring the response of a cell system exposed to a sample. The denotation "estrogen" summarises different natural and synthetic estrogens (e.g. estrone (E1), 17ß-estradiol (E2) and 17α-ethinylestradiol (EE2)) as well as their numerous conjugates (e.g. glucuronides, sulphates, disulphates, e.g. estrone-3-sulphate (E1-3S)). estrogen conjugates are relevant in this context because their receptor-binding potency is much lower than that of estrogens. However, they can be transformed to estrogens by deconjugation. Their potential estrogenic activity cannot be detected with bioassays prior to deconjugation. Apart from estrogens, the estrogen receptor can also be activated by non-steroidal substances that imitate estrogens and bind to the estrogen receptor (xenoestrogens).

In an attempt to standardise the effects of the different estrogens in receptor-based assays, their relative estrogenic potency (REP) has been defined in relation to the effect of E2 in the respective assay (e.g. [23]). The REP is bioassay specific and can vary for one bioassay between different laboratories [11].

Substances stimulating the estrogen receptor are called agonists, while substances with an inhibiting effect are called antagonists. Various natural and synthetic substances are known to have antagonistic effects in receptor-based estrogen assays [24]. In environmental samples, such as manure, soil or drainage water, agonistic and antagonistic effects may be modulated by matrix effects, caused by adsorption to particles or by chemical binding to colloidal organic substances [25].

estrogenic activity in manure samples

The estrogenic activity of the manure applied at the Guettingen field site varied from 201 to 2675 $\mu g/m^3$ EEQ (ER-CALUX) and from 955 to 7888 $\mu g/m^3$ EEQ (yeast estrogen screen (YES)) (Table 4). The highest EEQ (YES)-value was more than 20 times higher than the lowest EEQ (ER-CALUX)-value. The EEQ in all manure samples was 2.9 to 14.2 times higher if measured with the YES than with the ER-CALUX. In the

Table 4 Composition, date of application, applied load and EEQ of three manure mixtures (M1–M3) applied at the Guettingen field site during this study

No.	Date of manure application	Manure composition/substrate extracted	Load (m^3/ha)[a]	EEQ (ER-CALUX) (μg/m^3)	EEQ (YES) (μg/m^3)
M1	April 21, 2010	50 % cattle/50 % chicken manure	30	201	955
M2	April 28, 2010	100 % cattle manure	20	2675	7888
M3	June 15, 2010	66 % cattle manure/33 % water	30	480	6816
K1	–	Ultra pure water	–	<LOD	<LOQ
K2	–	Ultra pure water spiked 2 μg/kg E2	–	1078	611
K3	–	M2, spiked with 2 μg/kg E2	–	2909	9856

K1 to K3 are procedural controls. The EEQ was calculated assuming a manure density of 990 kg/m^3
[a]According to W. Vogt (personal communication)

control K2, EEQ (ER-CALUX) and EEQ (YES) differed only by a factor of 0.6.

In comparison to values reported in literature, our measurements are in the lower range. Dyer et al. (2001, cited by Hanselmann et al. [26]) measured 3300 ng/kg (wet weight) of E2 in liquid dairy manure, which is equivalent to 3333 μg/m^3. Based on earlier work of Raman (2004), Johnson et al. [7] calculated a 17ß-estradiol equivalent of 31 μg/kg for typical dairy cow manure, corresponding to 31,313 μg EEQ per m^3 of manure.

The composition of manure depends on different factors: storage time influences the concentration of estrogens and the distribution between the different estrogens [27]. Age structure of the cattle herd, the ratio of pregnant cows, livestock husbandry conditions (grazing vs. confinement) and the use of feed additives may influence the amount of estrogens in manure. Finally, farmers mix manure with water or other types of manure (Table 4), according to their experience, needs, manure type and availability. In this study, EEQ values of manure samples were systematically higher in the YES than in the ER-CALUX. This finding will be discussed in the "Oestrogenic activity in drainage pipe water" section.

estrogenic activity in drainage pipe water

The conditions during the five field campaigns are summarised in Table 5. estrogenic activities (EEQ) found at the drainages "Shaft" and "Outlet" are summarised in Fig. 4 and Fig. 5, respectively. The LOD of ER-CALUX and YES are reported in the "Analysis of estrogenic activity" section.

In field campaign FC1 (2 days after manure application on pasture "East"), both ER-CALUX and YES recorded a slight increase in EEQ at "Shaft", close to the LOD of both assays. At "Outlet", where the drainage waters of the whole system merge, the EEQ values were below the level of quantification (ER-CALUX) and below the level of detection (YES). Outflow at "Shaft" was only moderately increased by the 4.6 mm of rain. Soil moisture tension in the lower part of the soil remained high and did not drop, as it would be expected during an infiltration event (Fig. 3). This indicates that the upper soil layer was able to absorb the rainwater and no significant washout of manure constituents took place.

Field campaign FC2 (14 days after manure application on pasture "East") was conducted on a sunny day between 11:30 and 15:30. A rain event had been forecasted for this

Table 5 Frame conditions and oestrogenic activity of the five field campaigns

ID	Duration of FC, date, time	Days since manure application	Rain during FC (mm)	Soil moisture tension at 12.5 cm (hPa)		Max. EEQ (ER-CALUX) ± MU (ng/l EEQ)[a]		Max. EEQ (YES) ± SD (ng/l EEQ)[b]	
				Start	End	Shaft	Outlet	Shaft	Outlet
FC1	24 h April 30, 16:00 to May 1, 16:00	2 days (East)	4.6	820	720	0.054 ± 0.01 (LOD 0.0008)	<LOQ (0.023) (LOD 0.0008)	0.14 ± 0.02	n.d.
FC2	4.5 h May 12, 11:00 to May 12, 15:30	14 days (East)	0	40	50	4.7 ± 1.22 (LOD 0.0008)	0.15 ± 0.04 (LOD 0.0008)	7.98 ± 2.65	0.69 ± 0.21
FC3	24 h May 26, 16:00 to May 27, 16:00	28 days (East)	18.8	510	105	0.74 ± 0.19 (LOD 0.0008)	10 ± 2.60 (LOD 0.0008)	2.9 ± 0.22	11.07 ± 2.31
FC4	24 h June 17, 16:00 to Jun 18, 16:00	2 days (East + West)	45.1	260	70	9 ± 2.34 (LOD 0.0008)	0.34 ± 0.09 (LOD 0.0008)	14.08 ± 1.05	0.65 ± 0.02
FC5	4 h June 24, 11:20 to June 24, 15:20	9 days (East + West)	0	355	380	0.049 ± 0.01 (LOD 0.0008)	0.048 (LOD 0.0008)	n.d.	n.d.

MU measurement uncertainty, 26 %, *SD* standard deviation, *n.d.* not detectable
[a]Data as reported by BDS
[b]Data as reported by Ecotox Centre

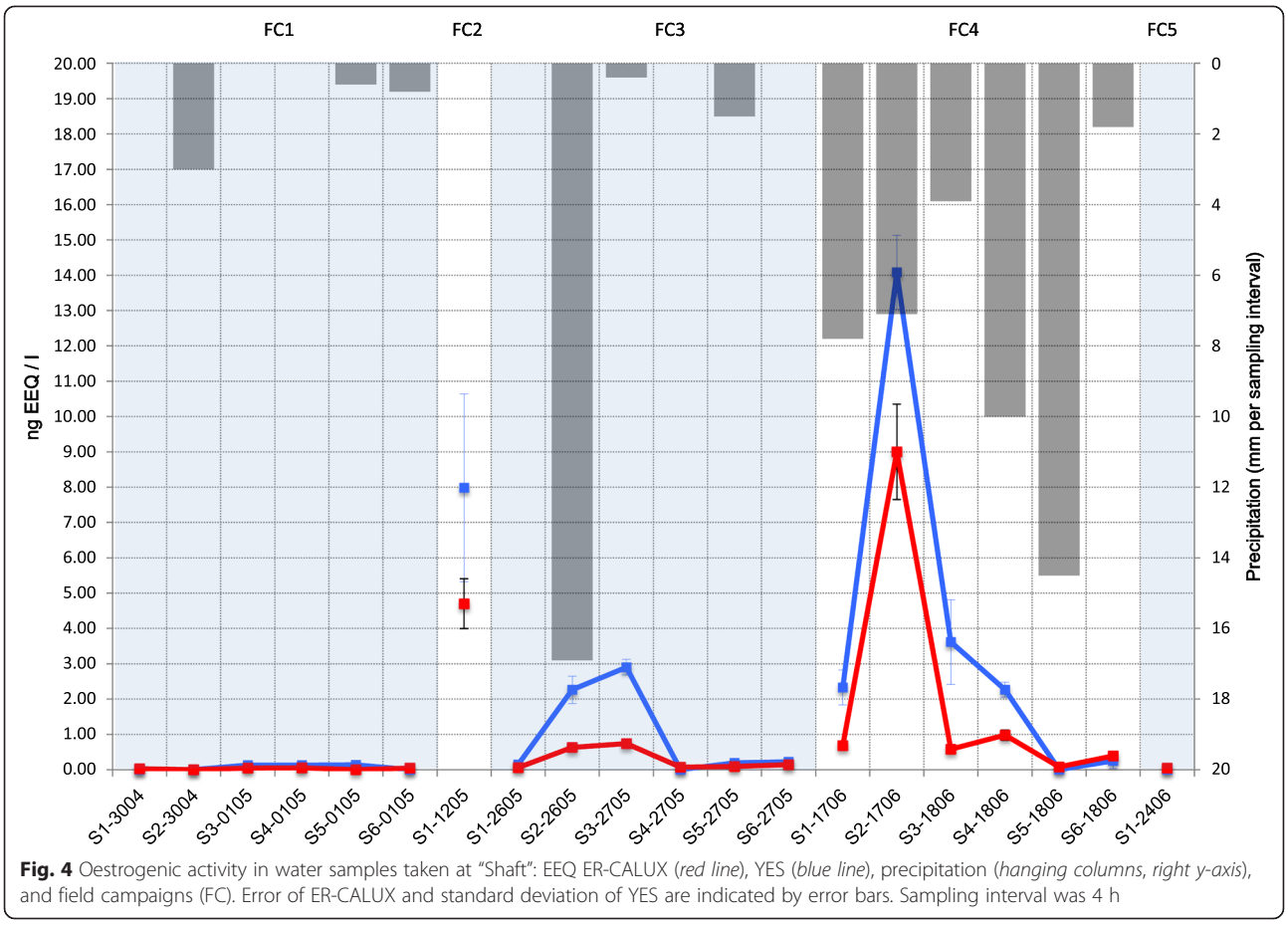

Fig. 4 Oestrogenic activity in water samples taken at "Shaft": EEQ ER-CALUX (*red line*), YES (*blue line*), precipitation (*hanging columns*, *right y-axis*), and field campaigns (FC). Error of ER-CALUX and standard deviation of YES are indicated by error bars. Sampling interval was 4 h

afternoon, and when it did not occur, we intended to use the FC as a dry weather reference. In the 12 days before FC2, a total of 67.9 mm of rain was recorded. The last rain fell 17.5 h before FC2. Outflow at "Shaft" was moderately stable during FC2, and the soil moisture tension of the upmost soil layer indicates near water-saturation of the soil (Fig. 3). Regarding the stable hydraulic conditions, EEQ values found at "Shaft" were unexpectedly high. The estrogenic activity may originate from cowpats and cow urine, since the grazing period had just ended a day before. Another possible explanation is that estrogens from manure application bound to soil particles were released due to the water-saturation of the soil, which is usually combined with anaerobic conditions.

Field campaign FC3 was conducted 28 days after the last manure application on pasture "East". The largest share of the rain fell within 30 min around 20:00. Outflow at "Shaft" increased within 30 min after the onset of the rainfall and decreased rapidly within an hour after its end. The soil moisture tension of the top soil dropped sharply during FC3, from rather dry to almost saturated (Fig. 3). EEQ values at "Shaft" were low before the rainfall, showed an increase between 20:00 and 4:00 to a maximum of 0.74 ± 0.19 ng/l (ER-CALUX) and 2.9 ± 0.22 ng/l (YES), and dropped to very low values right after that (Fig. 4). The origin of these estrogenic compounds must be located in the direct catchment of "Shaft". If manure or cow excreta were the origin, the estrogenic compounds must have been stable in the soil for at least 14 days—the time since the end of the grazing period. Surprisingly, at "Outlet", EEQ values increased to 11.07 ± 2.31 ng/l (YES) and 10 ± 2.60 ng/l (ER-CALUX) between 0:00 and 8:00 (Fig. 5). An explanation for these high EEQ peaks at "Outlet" is manure application by farmers in the upper parts of the catchment of "Outlet" on the afternoon just before FC3, followed by direct washout of manure from these pastures by rainfall.

Field campaign FC4 was conducted 2 days after manure application on the catchment of "Shaft", following the grass harvest. Abundant rain fell during FC4, as part of a cold front. Drainage pipe outflow increased from the usual low values around 0.1 l/s to more than 4 l/s during FC4 and only declined slowly afterwards. Soil moisture tension of the top soil dropped sharply during FC4, from moderately dry to almost saturated (Fig. 3). All samples taken during FC4 were brownish in colour. The sample taken from 20:00 to 24:00 at "Shaft" strongly smelled like manure, and the others a little less but still

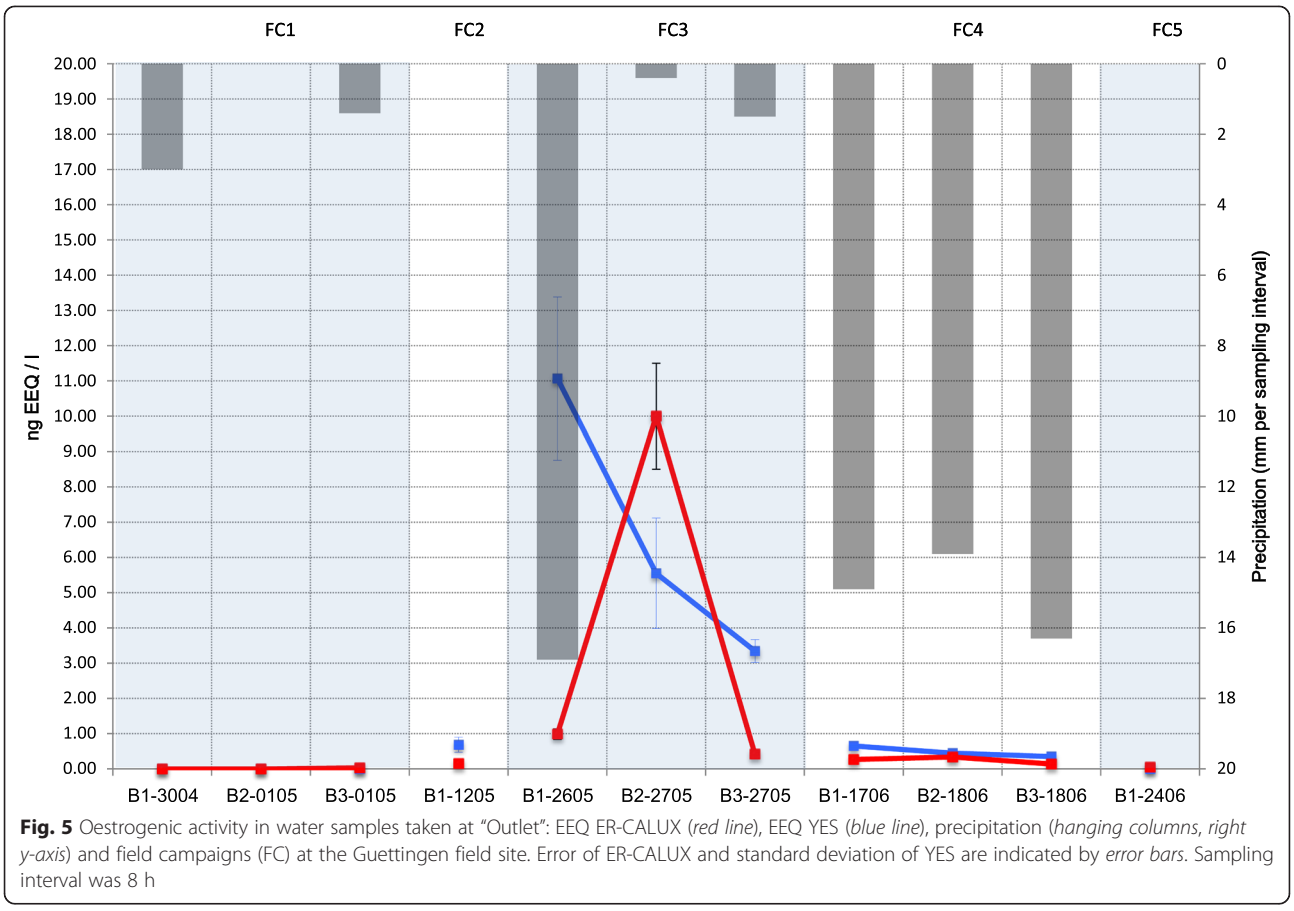

Fig. 5 Oestrogenic activity in water samples taken at "Outlet": EEQ ER-CALUX (*red line*), EEQ YES (*blue line*), precipitation (*hanging columns*, *right y-axis*) and field campaigns (FC) at the Guettingen field site. Error of ER-CALUX and standard deviation of YES are indicated by *error bars*. Sampling interval was 8 h

perceivably. The EEQ at "Shaft" reached a maximum of 9 ± 2.34 ng/l (ER-CALUX) and 14.08 ± 1.05 ng/l (YES) in this sampling period and rapidly dropped to lower values afterwards (Fig. 4). The high EEQ peaks after the onset of the rain can be explained by direct washout of manure-borne estrogenic compounds by rainfall. At "Outlet", outflow was torrential during FC4, leading to a dilution of the estrogenic load, resulting in EEQ values of 0.34 ± 0.09 ng/l (ER-CALUX) and 0.65 ± 0.02 ng/l (YES) (Fig. 5).

Field campaign FC5 was conducted 9 days after manure application on a sunny day, in a phase of declining outflow following FC4, and more than 4 days after the last rainfall. Outflow at "Shaft" was 0.3 l/s during all of FC5. The EEQ (ER-CALUX) was close to the level of detection. With the YES, no EEQ was detectable.

The three procedural blanks (K1-0705, K3-0705, K1-0306) showed no detectable EEQ in the YES. In the ER-CALUX, the procedural blank was below the level of quantification (LOQ, <0.016 ng/l) in two of the three cases, and 0.035 ± 0.009 ng/l in one case. The five procedural blanks spiked with 2 ng/l E2 had a mean EEQ of 0.81 ± 0.30 ng/l (ER-CALUX) and 1.94 ± 0.53 ng/l (YES). The procedural blank spiked with 10 ng/l E2 had an EEQ of 5.5 ± 1.43 ng/l (ER-CALUX) and 20.1 ± 0.7 ng/l (YES) (see Table 6).

Throughout this study, the EEQ (ER-CALUX) values in water and manure samples were consistently lower than those measured with the YES. Several studies have already shown that different ER-bioassays lead to different EEQ values when analysing the same environmental sample. Reasons for these differences are known and most likely due to the differences of the assays. In the specific case of agricultural estrogens, one explanation is the difference in sensitivity of the two assays towards estrone. estrone is 10 times less potent in the ER-CALUX (REP of 0.02, E2 has an REP of 1) than in the YES (REP of 0.265). This may already explain the continuously lower EEQ values measured in the ER-CALUX, as large parts of the estradiol present in manure is oxidised to estrone within hours [28, 13]. However, the lower EEQ values of the ER-CALUX could have also been caused by an unknown antagonistic effect. The scope of this study does not allow a final conclusion.

The field campaigns showed that, when heavy rainfall occurred after manure spreading, EEQ values in drainage pipe water at "Shaft" and "Outlet" reached peak values higher than 10 ng/l during short periods of 4–8 h.

Table 6 Water samples, procedural controls: expected and measured EEQ

Sample	Procedural control processed with SPE	SPE processed vol. (l) (conc. factor)	Expected EEQ (ng/l)	EEQ (ER-CALUX) ± MU (ng/l)[a]	EEQ (YES) ± SD (ng/l)[b]
K1-0705	HPLC-grade water	0.991(1713 x)	0	<LOQ (0.016) (LOD 0.008)	n.d.
K2-0705	HPLC-grade water, spiked to 2 ng/l with E2	0.967(1672 x)	2	0.75 ± 0.195 (LOD 0.008)	1.72 ± 0.78
K3-0705	9:1 HPLC-grade water : acetone	0.350(601 x)	0	<LOQ (0.079) (LOD 0.008)	n.d.
K1-0306	HPLC-grade water	0.977(1656 x)	0	0.035 ± 0.009 (LOD 0.008)	n.d.
K2-0306	HPLC-grade water, spiked to 2 ng/l with E2	0.843(1425 x)	2	0.45 ± 0.117 (LOD 0.008)	1.81 ± 0.15
K1-0107	HPLC-grade water, spiked to 2 ng/l with E2	0.838(1425 x)	2	1.3 ± 0.338 (LOD 0.008)	1.91 ± 0.05
K1-0809	HPLC-grade water, spiked to 10 ng/l with E2	0.992(1993 x)	10	5.5 ± 1.43 (LOD 0.013)	20.1 ± 0.7
K2-0809	HPLC-grade water, spiked to 2 ng/l with E2	0.996(1996 x)	2	0.77 ± 0.2 (LOD 0.013)	2.83 ± 0.18
K3-0809	HPLC-grade water, spiked to 2 ng/l with E2	0.995(2003 x)	2	0.8 ± 0.208 (LOD 0.013)	1.42 ± 0.1

MU measurement uncertainty, 26 %, *SD* standard deviation, *n.d.* not detectable
[a]Data as reported by BDS
[b]Data as reported by Ecotox Centre

Different timing or different practices of neighbouring farmers concerning manure application can lead to a series of EEQ peaks in the receiving stream: during FC3, at "Outlet", EEQ values >10 ng/l were detected, while EEQ at "Shaft" remained much lower. This was due to manure application in the upper part of the catchment of "Outlet". In contrast, during FC4, the EEQ was much higher at "Shaft" than at "Outlet". Furthermore, in two of our five field campaigns, estrogenic activity was found in the drainage pipe water at "Shaft" without an apparent link to manure application directly before the field campaign (FC3), or even rainfall during the field campaign (FC2). This coincides with the results of Kjaer et al. [13]. They found that a washout of estrogens through drainage pipes can still occur months after manure application, and related it to soil conditions. Gall et al. [29] observed that "significant export (of hormones) was found during the spring prior to the addition of animal wastes". This suggests that "soil may act as a long-term reservoir for E2 in the environment" [30].

Contribution of phytoestrogens to total estrogenic activity

In Fig. 6, EEQ (ER-CALUX) values of eight selected samples are compared to the calculated estrogenicity (calEEQ) of six phytoestrogens measured in this study. In six out of eight samples, these phytoestrogens explain less than 13 % of the EEQ (ER-CALUX). Under peak outflow conditions, as they were found at "Outlet" in B2-2705 and at "Shaft" in S2-1706, the calEEQs of these phytoestrogens explain less than 3 % of the total EEQ (ER-CALUX). The unexpectedly high EEQ value in sample S1-1205, which was sampled under low-outflow and dry weather conditions, thus cannot be attributed to phytoestrogens.

Conclusions

The results of this study can be summarised as follows:

- EEQ values in manure vary greatly.
- Under base flow conditions, the EEQ values in drainage water are either below the level of detection (LOD) or in the lower range of all reported measurements.
- Manure-borne estrogenic activity in drainage pipe waters can temporarily reach EEQ values higher than 10 ng/l for 4–8 h.
- The highest peak values were found during heavy rainfall events, 1–2 days after manure spreading.
- Two neighbouring drainage catchments can show different patterns regarding EEQ peak values in drainage water.
- High EEQ peaks in drainage water can also occur weeks after manure application. In two cases, we found high EEQ values at "Shaft" 14 days (FC2) and 28 days (FC3) after the last manure application. This supports the hypothesis of Schuh et al. [18] that "soil may act as a long-term reservoir for E2 in the environment".
- Washout in these cases seems to be linked with soil moisture tension.
- Grab water samples from drainages at base flow conditions are therefore not useful for assessing the EEQ load from a drainage catchment.
- Peak EEQ concentrations will often have a time coincidence with peak runoff in the whole catchment and thus will be diluted, as it was observed in FC4. This will lower the concentration to which organisms are exposed.

Our results are supported by the findings of Gall et al. [29] in their study on hormone export from a "tile-

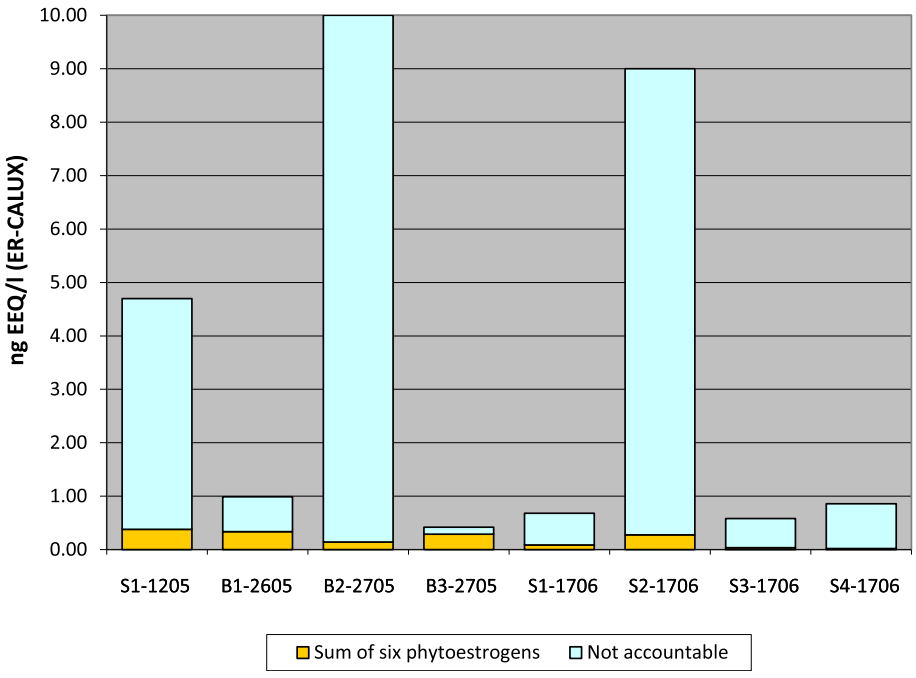

Fig. 6 Comparison of the EEQ (ER-CALUX) of eight samples from "Shaft" (*S*) and "Outlet" (*B*) with the calculated total oestrogenicity of six phytooestrogens measured in the sample extracts with LC/MS/MS

drained agroecosystem receiving animal wastes". They found that "higher hormone concentrations generally occurred during discrete periods of increased flow", "high flow rates often were associated with a disproportionately high hormone flux" and "hormone fluxes were highest during storm events that occurred shortly after animal waste applications".

Organisms (including their eggs, embryos and hatchlings) living in streams in agricultural areas with manure application and a high percentage of drained area may thus be exposed to numerous manure-borne EEQ peak concentrations per year. Fish eggs and fish embryos in the sediment would inevitably be exposed to such EEQ peak concentrations, because they are stationary. Brown trout (*Salmo trutta fario* L.) is a typical fish of small streams in the Swiss lowlands, whose embryogenesis may be affected by this. It spawns between October and January [31]. At the typical winter water temperatures of 4–5 °C, development of the eggs takes 90–100 days, and ends between January and April. Brown trout populations have been declining in Switzerland since 1980 [32].

Schubert et al. [33] examined the sensitivity of brown trout embryos (*Salmo trutta fario* L.) to "environmentally relevant concentrations" of E2 in the time between fertilisation and hatch (70 days). They exposed the fish embryos to transient E2 concentrations of 3.8 and 38.0 ng/l E2 for 2 h. Four scenarios were investigated: exposure (a) directly after fertilisation, (b) at "eyeing stage", (c) weekly until hatch, and (d) bi-weekly until hatch. Their conclusion was that "even a single, transient E2 exposure during embryogenesis" has "significant effects on brown trout development".

In the Canton of Thurgau, manure is applied four to six times per year on managed pastures. The first application in March has a possible time coincidence with the hatching period of brown trout, while the last in October or November has a time coincidence with spawning. Since farmers have individual strategies for manure application, it is not practised in a synchronised manner, which potentially increases the number of EEQ peak concentrations in a given stream location. Development of brown trout embryos also falls into a season with low vegetation activity, low soil temperature and low general biological activity. Degradation of a postulated EEQ reservoir in the soil would therefore be slower than in spring or summer, and EEQ washout-events in connection with rainfall may even be more likely than in warmer seasons. If embryogenesis of brown trout should be negatively affected by manure-borne EEQ peaks, this may be an additional reason for declining brown trout populations in Switzerland.

There are also indications that pulses of estrogens in low concentrations can cause effects in juvenile or adult fish exposed to them. Hyndman et al. [34] examined the effects of differential timing of exposure

with E2 on a range of fathead minnow (*Pimephales promelas*) biomarkers in a laboratory study. They found that the ability of treatment male fish to hold nest sites in direct competition with control males was sensitive to E2 exposure. Labadie and Budzinski [35] concluded that juvenile male turbots (*Psetta maxima*) are susceptible to hormonal imbalance as a consequence of short-term exposure to environmentally relevant 17α-ethinylestradiol (EE2) levels.

The postulated "long-term reservoir (…) in the soil" [19] will probably contain natural estrogens in conjugated and deconjugated forms, with specific REP for every substance. Urine of pregnant cattle for example contains estrone-3-sulphate (E1-3S), the dominating conjugated form of E1, which has a very low relative potency in the E-screen assay (0.000012, [10]). It can be expected that E1-3S will be deconjugated over time, but it is, to our knowledge, unknown how fast this process proceeds. If conjugated estrogens are "hydrolysed to their free forms in the environment, they could contribute additional estrogenic activity" [10]. Degradation of estrogens is reportedly slowed down by anaerobic conditions [17], sheep urine [15] and by DOC from manure [16]. It can be expected that these factors will influence the half-life-time of estrogen conjugates and thus the size and estrogenic activity of the "long-term reservoir" as well. This aspect deserves further investigation.

Effects observed in bioassays display the overall estrogenicity of a sample and cannot easily be associated to specific substances. In this study, we could not include chemical measurements of natural estrogens. Thus, a complete toxicity identification evaluation was not possible. Further research is therefore necessary to verify and understand the postulated "long-term reservoir" and link specific estrogens to the EEQ values measured with bioassays.

Methods

One drainage pipe from a cattle pasture was monitored for 73 days in spring 2010 (April 16–June 28). A set of physical and chemical parameters was monitored continuously (see "Contribution of phytoestrogens to total estrogenic activity" section). During three typical storm events and two reference periods without rainfall, water samples were collected in consecutive intervals at two sampling sites (see "Estrogenic activity of manure and drainage water samples" section). estrogenic activity in the samples was determined with the ER-CALUX® and the YES bioassays. Manure-related nutrients were determined using field equipment.

Sampling location

This study was performed at a 30-ha dairy farm in Guettingen, Switzerland, with about 60 dairy cows, one bull and ten calves. The stocking density of about 2 animal units/ha was slightly above the Swiss average of 1.71 [36]. The study area is located on a gently north-sloping hillside facing the Lake of Constance. The soil developed on glacial till and can be characterised as well weathered, slightly acidic brown earth. It is influenced by groundwater in the lower parts of the horizon (C. Boesiger, ZHAW Bachelor's thesis 2010, unpublished), which was the reason for draining it. Guettingen lies 440 m above sea level and has an average precipitation of 916 mm per year (source: MeteoSwiss). The climate at Guettingen can be characterised as temperate oceanic to humid continental [37].

Monitoring and sampling was done in the pipe outlet draining the dairy cow pastures "West" and "East" (sampling site "Shaft", Fig. 1). In addition, samples were collected where the whole drainage system enters the stream Othmarsbach (sampling site "Outlet"). Drainage at "Shaft" originated exclusively from the two cow pastures. Water at "Outlet" was a mixture of drainage pipe water from "Shaft", road runoff and the much larger area of drained pastures uphill. It cannot be excluded that the water at "Outlet" contained traces of domestic wastewater. The exact contribution of drainage water from "Shaft" to the outflow at "Outlet" is unknown.

The GIS record of the drainage system shown in Fig. 1 is probably incomplete. According to W. Vogt, the drainage pipes collecting at "Shaft" (built around 1950) lie at an estimated soil depth of about 1–1.50 m and have a total length of about 40–50 m. Based on these incomplete data, the size of the catchment drained at "Shaft" was roughly estimated at about 500 m^2. Additional surveying was not possible within this project. Soil parameters were examined at the sampling point "Soil", between the two drainage pipes.

On average, manure is spread four to six times per year on the farm's pastures, using a drag hose device. Cow manure is sometimes mixed with pig and chicken manure from neighbouring farms. The grass of the pastures is harvested or used by direct grazing at different times of the year (personal communication of the farmer).

Sampling

As a general precaution, all material that came into contact with the samples was pre-rinsed three times with ultra-clean acetone (puriss.p.a., Sigma-Aldrich 00570). All dilutions were made with HPLC-grade water (J.T.Baker 4218). Contact of the sample with plastics was avoided as much as possible.

Manure samples were collected with a scoop on the day of manure application and transferred directly to 1-litre glass bottles. They were immediately deep-frozen until further processing in the lab.

During rainfall events, water samples were collected in time-dependant steps with auto-samplers (ISCO). Sampling generally started before the rainfall events, and

continued for 24 h in consecutive sampling intervals. Sampler settings and sampling intervals are summarised in Table 7.

Five FC were conducted (see Fig. 2). FC1 (30.4./1.5.), FC3 (26.5./27.5.) and FC4 (17.6./18.6.) started at 16:00 and lasted for 24 h, equalling six sampling intervals at "Shaft", and three sampling intervals at "Outlet", respectively. FC2 (12.5., 11:30–15:30) and FC5 (24.6., 11:20–15:10) lasted for 4 h, or 1 sampling interval at each location. They were conducted during dry weather conditions.

After each sampling interval, the collected samples were removed from the auto-samplers. Three 600-ml subsamples were taken from each sample and stored in 1-litre glass bottles. The remaining volume was analysed for chemical parameters. The bottles were labelled with "S" (Shaft) or "B" (Outlet), the number of the interval, the number of the subsample and the date. All samples were deep-frozen at −18 °C within 2 h after collection.

Sample preparation and extraction

The frozen samples were transported to the lab and stored there at −18 °C until preparation.

For the extraction of manure samples, the slightly modified method of Zhao et al. [38] was used: manure samples were thawed in a water bath at 10 °C. They were agitated vigorously to resuspend particles. Subsequently, 20 ml of raw manure was taken and mixed with 80 ml of 1 M NaOH and allowed to settle for 30 min. Of the supernatant, 6 ml was transferred to a fresh vial with 6 ml of chloroform. The vial was vortexed two times for 20 s and phase separation was awaited. An aliquot of 5 ml of the aqueous phase was transferred to another vial, neutralised with 190 µl of acetic acid (90 %) and run through solid-phase extraction (SPE) as described below.

For the extraction of water samples, the samples were thawed in a water bath at 10 °C and immediately adjusted to pH 3 (±0.1) with 1 M HCl. Thereafter, 1.2 l of each sample was filtered through 1 µm glass fibre filters (Whatman GF/F). SPE Cartridges (200 mg LiChrolut EN, Merck) were preconditioned by subsequently adding 2 ml of hexane (>99 %, Sigma-Aldrich), 2 ml of acetone (puriss.p.a, Fluka), 3 × 2 ml of methanol (>99 %, Sigma-Aldrich) and 3 × 2 ml of HPLC-grade water (J.T. Baker), and by letting the solvents run through the cartridge by gravity. After the last preconditioning step, the lower valve of the SPE cartridge was closed; it was filled with HPLC-grade water up to the upper rim and the sample bottle was connected with a Teflon-coated tube. The filtered samples were then pulled through the SPE cartridges under vacuum, within about 60–90 min. After completion, SPE cartridges were dried under N_2 flow (0.4 bar) and stored at −18 °C if necessary. Elution was conducted with 4 × 1 ml acetone (puriss.p.a, Fluka). The extracts were evaporated under N_2 flow, redissolved in 0.5 ml absolute ethanol (puriss.p.a, Sigma), portioned and stored in silanised amber vials (Supelco 27072-U). The portions were sent cooled and via express mail to BDS (0.2 ml, Amsterdam, The Netherlands) for the ER-CALUX® assay, and to the Ecotox Centre Eawag/EPFL (0.3 ml, Duebendorf, Switzerland) respectively, for the YES-assay. The weight of the samples was recorded at each step of the procedure, and concentration factors for each sample were calculated based on these records. The mean of the concentration factors was 1615 (standard deviation 330).

To assess the accuracy of the SPE, three procedural controls (K1–K3) were processed along with the manure samples (see Table 4). Nine procedural controls (HPLC-grade water without or with acetone, unspiked or spiked) were processed along with the water samples (Table 6). The extracts were analysed on estrogenic activity as described in "Analysis of estrogenic activity" section.

Analysis of estrogenic activity

The ER-CALUX is an "estrogen-receptor mediated, chemical-activated luciferase reporter gene-expression assay" based on human U2OS cells with an exogenous hERα receptor [39]. Estrogenic activity of a sample is quantified by using the amount of luciferase activity after 24 h of exposure.

In this project, all ER-CALUX measurements were commissioned to the ISO/IEC 17025-accredited company BioDetectionServices (BDS, Amsterdam, The Netherlands). BDS received 0.2 ml of water extracts (redissolved in ethanol) for analysis. These extracts were evaporated by them, redissolved in 25 µl DMSO and used for analysis in the ER-CALUX. All extracts and reference compounds were analysed in triplicates. In the ER-CALUX, "only dilutions that are negative in the cytotoxicity test" are "used for quantification of the response" [16]. Based on sample-specific SPE concentration factors provided by us, BDS reported (a) the calculated EEQ in the matrix (in ng 17β-estradiol equivalents per litre of water), (b) the

Table 7 Settings of the ISCO auto-samplers at "Shaft" and "Outlet"

Site	Sampler	Duration of sampling interval	Number of bottles in sampler	Volume per time step	Total sample volume after interval
"Shaft"	ISCO 6712 Portable Sampler	4 h	4	0.1 l/10 min	2.4 l
"Outlet"	ISCO GLS Compact Composite Sampler	8 h	1	0.125 l/25 min	3.0 l

measurement uncertainty and (c) the level of detection (LOD) for every batch of measurements. Data were regarded "quantifiable between the limit of quantification (LOQ) and the EC50", and "only results within this range are included in the final results" (BDS reports). At BDS, all measurements with a standard deviation higher than 15 % are repeated as part of the regular laboratory routine. Measurement uncertainty was reported as 26 % for all measurements. The LOD of the ER-CALUX differed between batches but was always 0.017 ng/l E2 or lower.

The YES, an estrogen-inducible expression system, is described in detail by Routledge and Sumpter [40]. In brief, the yeast (*Saccharomyces cerevisiae*) genome carries a stably integrated DNA sequence of the human estrogen receptor (hERα). Yeast cells also contain expression plasmids carrying estrogen responsive elements, regulating the expression of the reporter gene lacZ (encoding the enzyme β-galactosidase). Thus, when an active ligand binds to the receptor, β-galactosidase is synthesised and secreted into the medium, leading to a colour change of chromogenic substrate chlorophenol red β-d-galactopyranoside (CPRG) from yellow to red.

In this project, all YES measurements were commissioned to the Ecotox Centre Eawag/EPFL (Switzerland) and were measured as described by Rutishauser et al. [41]. Based on the sample-specific SPE concentration factors, the Ecotoc Centre reported (a) the calculated EEQ in the matrix (in ng 17β-estradiol equivalents per litre of water) and (b) the standard deviation for every batch of measurements. The LOD of the YES at the Ecotox Centre Eawag/EPFL has been reported as 0.02 to 0.1 ng/l E2 [42]. The yeast cells were provided by John Sumpter (Brunel University, Uxbridge, UK). The evaluation of the generated data by fitting a dose response curve was carried out with GraphPad Prism 5 Software (La Jolla, CA, USA). The results were expressed as EC50 (the concentration causing 50 % of the maximum effect) as well as EEQ (estrogen equivalent concentration). The fit provided the EC50 value and out of this, the EC10 and EEQ values were calculated.

Chemical analysis of phytoestrogens

Selected samples from FC2, FC3 and FC4 (S1-1205, B1-2605, B2-2705, B3-2705, S1-1706, S2-1706, S3-1806 and S4-1806) were analysed for phytoestrogens at Agroscope Reckenholz-Tänikon (ART) Research Station. For budget reasons, analysis of phytoestrogens was limited to six phytoestrogens common in Swiss rivers: daidzein, genistein, coumestrol, equol, formonetin and biochanin A (selection based on [43]). The frozen sample bottles were brought to ART, thawed, spiked with an isotope-labelled internal standard, solid-phase extracted and analysed with LC/MS/MS as described by Erbs et al. [44].

Calculated EEQs (calEEQ) of the analytically determined concentrations of the estrogenic compounds were determined by multiplying the concentration of each compound with its relative potency in the YES, and adding up the values for the compounds [5]. The numbers for the relative potencies of the phytoestrogens were taken from [23].

Chemical and physical parameters

All water samples were analysed in the field on selected chemical parameters using Hach-Lange test kits (Hach-Lange, Rheineck, Switzerland) and a portable Hach-Lange Xion spectrophotometer: NH_4-N (LCK 304), NO_3-N (LCK 339), PO_4-P (LCK 349), chloride (LCK 311), German degrees of hardness °dH (LCK 327). In addition, all samples were measured with a portable multi-probe Hach HQ40d (Hach-Lange, Rheineck, Switzerland) on electrical conductivity, pH and temperature. At "Shaft", electrical conductivity, pH, temperature and oxygen content were also recorded every 15 min with a Troll 9500 multi-parameter-probe (In-Situ Inc., Ft. Collins, CO, USA) from May 12 to June 28, 2010.

Monitoring of rainfall and outflow

Rainfall data were obtained from the SwissMetNet station at Guettingen. For measuring the drainage pipe outflow at "Shaft", the outflow was directed through a V-notch weir. Water levels behind the V-notch weir were measured every 30 min by a pressure transducer (Keller Drucktechnik, type: PR-36 X W, Winterthur, Switzerland) and transmitted once a day to a server using the cellular phone network. The outflow was calculated from a calibration equation, which was based on measurements at different outflows.

Soil parameters

The soil horizons were classified according to Swiss soil taxonomy [45]. Saturated hydraulic conductivity was determined with a constant head well permeameter in the field, using water stained with "Brilliant Blue" to visualise flow paths. Organic carbon content, grain size and volume of macropores (at 60 hPa) were determined on undisturbed samples in the lab. Soil moisture tension (SMT) was monitored with six Watermark probes (Irrometer, Riverside, CA, USA) dug into the ground, positioned in the middle of the three horizons (at 12.5, 25 and 50 cm depth) and measured every 60 min with a data logger. The manufacturer Irrometer considers a soil below 100 hPa as "saturated" and between 100 and 200 hPa as "adequately wet". Between 300 and 600 hPa, an agricultural soil should be irrigated, and above 1000 hPa, it is considered "dangerously dry for production" (Irrometer Company Inc., undated) [46]. More details on the determination of soil parameters are described in (C. Boesiger, ZHAW Bachelor's thesis 2010, unpublished).

Abbreviations
calEEQ: calculated estrogenicity; E1: estrone; E2: 17β estradiol; EE2: 17α-ethinylestradiol; EEQ: 17β estradiol equivalent; FC: field campaign; LOD: level of detection; LOQ: level of quantification; REP: relative estrogenic potency; SMT: soil moisture tension; YES: yeast estrogen screen.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AS conceived the study, acquired funding, guided the study design and coordination, analysed and interpreted the data and co-authored the manuscript. PK managed the YES measurements, participated in data analysis and interpretation and co-authored the manuscript. MK participated in study design, data analysis and co-authored the manuscript. All authors read and approved the final manuscript.

Acknowledgements
Thank you to the Swiss Federal Office of the Environment FOEN for funding this study. W. and A. Vogt generously offered help and hospitality during the field campaign. O. Feurer, C. Boesiger and P. de Buren (all ZHAW) made the field campaign possible by being willing to be on the go, irrespective of the mood changes of the local weather. M. Kneubühl and L. Petit Matile helped to measure and interpret the data. T. Mengesha (EAWAG) was directly involved in the YES measurements. R. Schönenberger and M. Suter (EAWAG) and C. Hörger (ART) were responsible for LC/MS/MS measurements of selected samples. H. Besselink did the ER-CALUX measurements. M. Faden provided one of the two ISCO samplers. Güttingen municipality provided the GIS map of the drainage system. Meteorological data have been provided by MeteoSwiss, the Swiss Federal Office of Meteorology and Climatology. Cordial thanks go to all of you for your help in this field study.

Author details
[1]Institute of Natural Resource Sciences, Zurich University of Applied Sciences, P.O. Box CH-8200, Waedenswil, Switzerland. [2]Ecotox Centre Eawag/EPFL, P.O. Box 611, CH-8600 Duebendorf, Switzerland. [3]Kanton Thurgau, Amt für Umwelt, Abteilung Gewaesserqualitaet und -nutzung, Bahnhofstrasse 55, CH-8510 Frauenfeld, Switzerland.

Received: 10 February 2015 Accepted: 5 June 2015

References

1. Tyler CR, Routledge EJ. estrogenic effects in fish in English rivers with evidence of their causation. Pure Appl Chem. 1998;70:1795–804.
2. Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. Widespread sexual disruption in wild fish. Environ Sci Technol. 1998;32:2498–506.
3. Vethaak AD, Rijs GBJ, Schrap SM, Ruiter H, Gerritsen A, Lahr J. Estrogens and xeno-estrogens in the aquatic environment of the Netherlands occurrence, potency and biological effects, Dutch National Institute of Inland Water Management and Waste Water Treatment (RIZA) and the Dutch National Institute for Coastal and Marine Management (RIKZ). RIZA/RIKZ-report no. 2002.001, ISBN 9036954010. 2002.
4. Christiansen L, Winther-Nielsen M, Helweg C. Feminisation of fish—the effect of estrogenic compounds and their fate in sewage treatment plants and nature, Danish Environmental Protection Agency, Environmental Project No. 729. 2002.
5. Vermeirssen EL, Körner O, Schönenberger R, Suter MJ-F, Burckhardt-Holm P. Characterization of environmental estrogens in river water using a three-pronged approach: active and passive water sampling and the analysis of accumulated estrogens in the bile of caged fish. Environ Sci Technol. 2005;39:8191–8.
6. Burki R. 2006, Environmental estrogens as endocrine disruptors in Swiss rivers: Assessment of exposure, effects on brown trout and interaction with other stressors, Ph.D. Thesis, Philosophisch-naturwissenschaftliche Fakultät der Universität Bern, Switzerland.
7. Johnson AC, Williams RJ, Matthiessen P. The potential steroid hormone contribution of farm animals to freshwaters, the United Kingdom as a case study. Sci Total Environ. 2006;362(1–3):166–78.
8. Lange IG, Daxenberger A, Schiffer B, Witters H, Ibarreta D, Meyer HHD. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. Anal Chim Acta. 2002;473(1–2):27–37.
9. Hanselmann TA, Graetz DA, Wilkie AW, Szabo NJ, Diaz CS. Determination of steroidal estrogens in flushed dairy manure wastewater by Gas-chromatography-mass spectrometry. J Environmental Quality. 2006;35:695–700.
10. Gadd JB, Tremblay LA, Northcott GL. Steroid estrogens, conjugated estrogens and estrogenic activity in farm dairy shed effluents. Environ Pollut. 2010;158:730–6.
11. Alvarez DA, Shappell NW, Billey LO, Bermudez DS, Wilson VS, Kolpin DW, et al. Bioassay of estrogenicity and chemical analyses of estrogens in streams across the United States associated with livestock operations. Water Res. 2013;47(10):3347–336.
12. Peterson EW, Davis RK, Orndorff AK. 17b-estradiol as an indicator of animal waste contamination in mantled Karst aquifers, J. Environ. Qual. 2000; 29: 826-34.
13. Kjaer J, Olsen P, Bach K, Barlebo HC, Ingerslev F, Hansen M, et al. Leaching of estrogenic hormones from manure-treated structured soils. Environ Sci Technol. 2007;41:3911–7.
14. Lucas SD, Jones DL. Biodegradation of estrone and 17ß-estradiol in grassland soils amended with animal wastes. Soil Biol Biochem. 2006;38:2803–15.
15. Lucas SD, Jones DL. Urine enhances the leaching and persistence of estrogens in soils. Soil Biol Biochem. 2009;41:236–42.
16. Stumpe B, Marschner B. Dissolved organic carbon from sewage sludge and manure can affect estrogen sorption and mineralization in soils. Environ Pollut. 2010;158:148–54.
17. Ying GG, Kookana ARS, Dillon P. Sorption and degradation of selected five endocrine disrupting chemicals in aquifer material. Water Res. 2003;37(15):3785–91.
18. Schuh MC, Casey FXM, Hakk H, DeSutter TM, Richards KG, KhanE, Oduor P. An on-farm survey of spatial and temporal stratifications of 17ß-estradiol concentrations. Chemosphere, 2010, doi:10.1016/j.chemosphere.2010.10.093.
19. Shappell NW, Elder KH, West M, Estrogenicity and nutrient concentration of surface waters surrounding a large confinement dairy operation using best management practices for land application of animal wastes, Environ. Sci. Technol. 2010;44:2365–2371.
20. Matthiessen P, Arnold D, Johnson AC, Pepper TJ, Pottinger TG, Pulman KGT. Contamination of headwater streams in the United Kingdom by estrogenic hormones from livestock farms. Sci Total Environ. 2006;367(2–3):616–30.
21. Hooda PS, Edwards AC, Anderson HA, Miller A. A review of water quality concerns in livestock farming areas. Sci Total Environ. 2000;250(1–3):143–67.
22. Liechti P. Methoden zur Untersuchung und Beurteilung der Fliessgewässer. Chemisch-physikalische Erhebungen, Nährstoffe. Bern: Umwelt-Vollzug Nr. 1005. Bundesamt für Umwelt; 2010. p. 44.
23. Coldham NG, Dave M, Sivapathasundaram S, McDonnell DP, Connor C, Sauer MJ. Evaluation of a recombinant yeast cell estrogen screening assay. Environ Health Perspect. 1997;105(7):734–42.
24. Fent K. 2007, Ökotoxikologie – Umweltchemie, Toxikologie, Ökologie, Thieme Verlag, Stuttgart.
25. Zhao S, Zhang P, Melcer ME, Molina JF. Estrogens in streams associated with a concentrated animal feeding operation in upstate New York. USA Chemosphere. 2010;79:420–5.
26. Hanselman TA, Graetz DA , Wilkie AC, Manure-Borne Estrogens as Potential Environmental Contaminants: A Review, Environ. Sc. Technol. 2003; 37: 5471-8.
27. Zheng W, Yates S, Bradford SA. Analysis of steroid hormones in a typical dairy waste disposal system. Environ Sci Technol. 2008;42:530–5.
28. Schlenker G, Müller W, Birkelbach C, Glatzel PS. Experimental investigations into the effect of Escherichia coli and Clostridium perfringens on the steroid estrone. Berl Munch Tierarztl Wochenschr. 1999;112:14–7.
29. Gall HE, Sassman SA, Jenkinson B, Lee LS, Jafvert C. Hormone loads exported by a tile-drained agroecosystem receiving animal manure wastes, Hydrological Processes, 2013, doi:10.1002/hyp.9664.
30. Schuh MC, Casey FXM, Hakk H, DeSutter TM, Richards KG, Khan E, et al. Effects of field-manure applications on stratified 17β-estradiol concentrations. J Hazard Mater. 2011;192(2):748–52.
31. Riedl C, Peter A. Timing of brown trout spawning in Alpine rivers with special consideration of egg burial depth. Ecol Freshw Fish. 2013;22:384–97.
32. Burkhardt-Holm P, Giger W, Güttinger H, Ochsenbein U, Peter A, Scheurer K, et al. Where have all the fish gone? The reasons why the fish catches in Swiss rivers are declining. Environ Sci Technol. 2005;39:441A–7A.
33. Schubert S, Peter A, Schönenberger R, Suter MJ, Segner H, Burkhardt-Holm P. Transient exposure to environmental estrogen affects embryonic development of brown trout (Salmo trutta fario). Aquat Toxicol. 2014;157:141–9.

34. Hyndman KM, Biales A, Bartell SE, SchoenfusS HL. Assessing the effects of exposure timing on biomarker expression using 17b-estradiol, Aquatic Toxicology 2010; 96: 264–72.
35. Labadie P, Budzinski H. Alteration of steroid hormone profile in juvenile turbot (Psetta maxima) as a consequence of short-term exposure to 17alpha-ethinylestradiol. Chemosphere. 2006;64(8):274–1286.
36. Bundesamt für Statistik, 2015, Nutztierhalter und Nutztierbestände 1985–2013, http://www.bfs.admin.ch/bfs/portal/de/index/themen/07/03/blank/data/01/03.html (3.2.2015).
37. Peel MC, Finlayson BL, McMahon TA. Updated world map of the Köppen-Geiger climate classification. Hydrol Earth Syst Sci. 2007;11:1633–44.
38. Zhao Z, Fang Y, Love NG, Knowlton KF. Biochemical and biological assays of endocrine disrupting compounds in various manure matrices. Chemosphere. 2009;74:551–5.
39. Van Der Linden SC, Heringa MB, Man HY, Sonneveld E, Puijker LM, Brouwer A, et al. Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. Environ Sci Technol. 2008;42:5814–20.
40. Routledge EJ, Sumpter JP. Estrogenic activity of surfactants and some of their degradation products assessed using a recom- binant yeast screen. Environ Toxicol Chem. 1996;15:241–8.
41. Rutishauser BV, Pesonen M, Escher BI, Ackermann GE, Aerni H-R, Suter MJ-F, et al. Comparative analysis of estrogenic activity in sewage treatment plant effluents involving three in vitro assays and chemical analysis of steroids. Environ Toxicol Chem. 2004;23:857–64.
42. Escher BI, Bramaz N, Quayle P, Rutishauser S, Vermeirssen ELM. Monitoring of the ecotoxicological hazard potential by polar organic micropollutants in sewage treatment plants and surface waters using a mode-of-action based test battery. J Environ Monit. 2008;10:622–31.
43. Hoerger C, Wettstein F, Hungerbühler K, Bucheli T. Occurrence and origin of estrogenic isoflavones in Swiss river waters. Environ Sci Tech. 2009;43(16):6151–7.
44. Erbs M, Hoerger C, Hartmann N, Bucheli TD. Quantification of six phytestrogens at the nanogram per liter level in aqueous environmental samples using 13C3-labeled internal standards. J Agric Food Chem. 2007;55:8339–45.
45. ART/ACW, (2008): Schweizerische Referenzmethoden der Forschungsanstalten Agroscope, Band 1, Methode KOM, E1.056.d, Version 2008.
46. Irrometer Company Inc. (undated), Irrometer/Watermark Sensor FAQ's, http://www.irrometer.com/faq.html#when (3.2.2015).
47. Bundesamt für Statistik, 2015, Bevölkerungsstand, http://www.bfs.admin.ch/bfs/portal/de/index/themen/01/02/blank/key/bevoelkerungsstand.html (3.2.2015).
48. Ying G-G, Kookana RS, Ru Y-J. Occurrence and fate of hormone steroids in the environment. Environ Int. 2002;28:545–51.
49. Brunner H, Conradin H, Gasser U, Kayser A, Lüscher P, Meuli R, et al. Klassifikation der Böden der Schweiz, Bodenkundliche Gesellschaft der Schweiz. 2010.