Electronic Supplementary Material (ESI) for Environmental Science: Processes & Impacts.

Supporting Information

Natural estrogens in surface waters of a catchment with intensive livestock farming in Switzerland

Daniela Rechsteiner,[a,b] Felix E. Wettstein,[a] Benjamin P. Warren,[b] Etiënne L. M. Vermeirssen,[c] Eszter Simon,[c] Manuel K. Schneider,[d] Juliane Hollender[b,e] and Thomas D. Bucheli*[a]

[a]Agroscope, Environmental Analytics, 8046 Zürich, Switzerland

[b]Institute of Biogeochemistry and Pollutant Dynamics, ETH Zurich, 8092 Zurich, Switzerland

[c]Swiss Centre for Applied Ecotoxicology, 8600 Dübendorf, Switzerland

[d]Agroscope, Forage Production and Grassland Systems, 8046 Zürich, Switzerland

[e]Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

*Corresponding author's e-mail: thomas.bucheli@agroscope.admin.ch

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S1 Information on the study area

Daily time-proportional water samples were collected of five Lake Baldegg tributaries for 30 days in February and March 2019. Animal densities in the studied catchments are listed in Table S1. Catchment and tributary characteristics are shown in Table S2. All cultivated crops in Lake Baldegg catchment in the vegetation period 2019 are listed in Table S4. They were divided in crops that were non-fertilized, mineral fertilized, organically or mineral fertilized and organically fertilized in February or March and their total area per catchment was calculated (Table S2).

The Canton of Lucerne provided the coordinates and livestock units (LSU) pig and cattle of every farm in the catchment of Lake Baldegg. The LSU of a tributary catchment was the sum of the LSU of all farms in the corresponding catchment. The LSU density per catchment equaled the total LSU per catchment divided by the organically fertilized agricultural area in February or March (Table S1). Stipulated by Swiss law, and according to common agricultural practice in Switzerland, most of the slurry spread in the catchments, originates from livestock animals housed in them.

Stoll et al.[1] characterized the connectivity of agricultural areas in the catchment of Lake Baldegg. For every tributary catchment, subplots of the agricultural area were classified into no connectivity to surface waters, low connectivity to surface waters, medium connectivity to surface waters, high connectivity to surface waters and drained areas. Classification was done according to Alder et al.[2] and Stoll et al.[1] In short, for every subplot of agricultural area (2m × 2m) the connectivity, meaning the overland flow distance to an extended drainage network (surface waters, drained roads, thalwegs), was estimated.[2] Subplots with a high probability to be drained (≥50%) were classified as drained areas.[1] Afterwards, the weighted mean percentage of agricultural area with high connectivity to surface waters was calculated for the total agricultural area of every tributary catchment (surface water-connected agricultural area) (Table S2).

The hydrological contribution describes the formation of fast flow components, i.e., preferential flow, saturation excess and Hortonian overland flow, of an agricultural area after a rain event. To identify hydrologically contributing agricultural areas a precipitation-discharge modelling approach was used. The model assumes that areas with the same topographic index[3] and soil have the same hydrological response. The area was divided in four hydrological response units, which reacted differently to rain events: poorly drained agricultural soils (Gleyic Cambisols and Eutric Gleysols), well drained agricultural soils (Eutric and Dystric Cambisols and Eutric Regosols), forest and urban areas.[4] In their modelling approach, poorly drained agricultural areas with a high probability to be drained (≥50%) were assumed to have a higher hydrological contribution.[1] Comparable to connectivity of agricultural areas to surface waters, the agricultural area was divided in five sub-classes of hydrological contribution after a small rain event (0-20 mm/d): very low hydrological contribution, low hydrological contribution, medium hydrological contribution, high hydrological contribution, very high hydrological contribution.[1, 4, 5] As previously described, the weighted mean percentage of agricultural area which shows a high hydrological contribution during a small rain event was estimated for the agricultural area of every sub-catchment (hydrologically contributing agricultural area) (Table S2).

Table S1: Livestock units cattle and pigs per hectare agricultural area that was organically fertilized in March for all monitored Lake Baldegg tributary catchments.

Tributary catchment	Livestock units cattle per hectare organically fertilized agricultural area [LSU/ha]	Livestock units pig per hectare organically fertilized agricultural area [LSU/ha]
Spittlisbach	3.3	1.6
Stagbach	1.6	1.3
Ron	1.6	1.1
Mulilbach	1.2	1.5
Hohibach	1.0	0.0

Table S2: Agricultural area related characteristics of monitored Lake Baldegg tributaries Spittlisbach, Stagbach, Ron, Mulibach, and Hohibach and their catchments.

Tributary	Catchment area [ha]	Organically fertilized agricultural area in March [ha]	Surface water-connected agricultural area[1] [%]	Hydrologically contributing agricultural area[1] [%]
Spittlisbach	361	204	43	54
Stagbach	932	644	43	59
Ron	2766	1811	48	61
Mulilbach	159	73	51	47
Hohibach	188	135	41	51

Mean discharge was relatively low in February and March 2019 in comparison to previous years. Furthermore, for some of the tributaries, i.e., Spittlisbach, Ron and Hohibach, mean discharge in February and March 2019 was even lower than all average monthly discharge rates from 1968 to 2018 (Table S3, Fig. S1).[6]

Table S3: Hydrological characteristics of monitored Lake Baldegg tributaries Spittlisbach, Stagbach, Ron, Mulibach, and Hohibach.

Tributary	Min. daily discharge March (1986-2018)[6] [m^3/s]	Mean daily discharge March (1986-2018)[6] [m^3/s]	Max. daily discharge March (1986-2018)[6] [m^3/s]	Mean daily discharge March 2019[6] [m^3/s]	Mean annual discharge (1986-2019)[6] [m^3/s]
Spittlisbach	0.001	0.067	1.440	0.022	0.059
Stagbach	0.018	0.146	4.870	0.083	0.132
Ron	0.090	0.576	9.950	0.218	0.548
Mulilbach	0.005	0.057	0.958	0.032	0.042
Hohibach	0.003	0.034	0.525	0.005	0.029

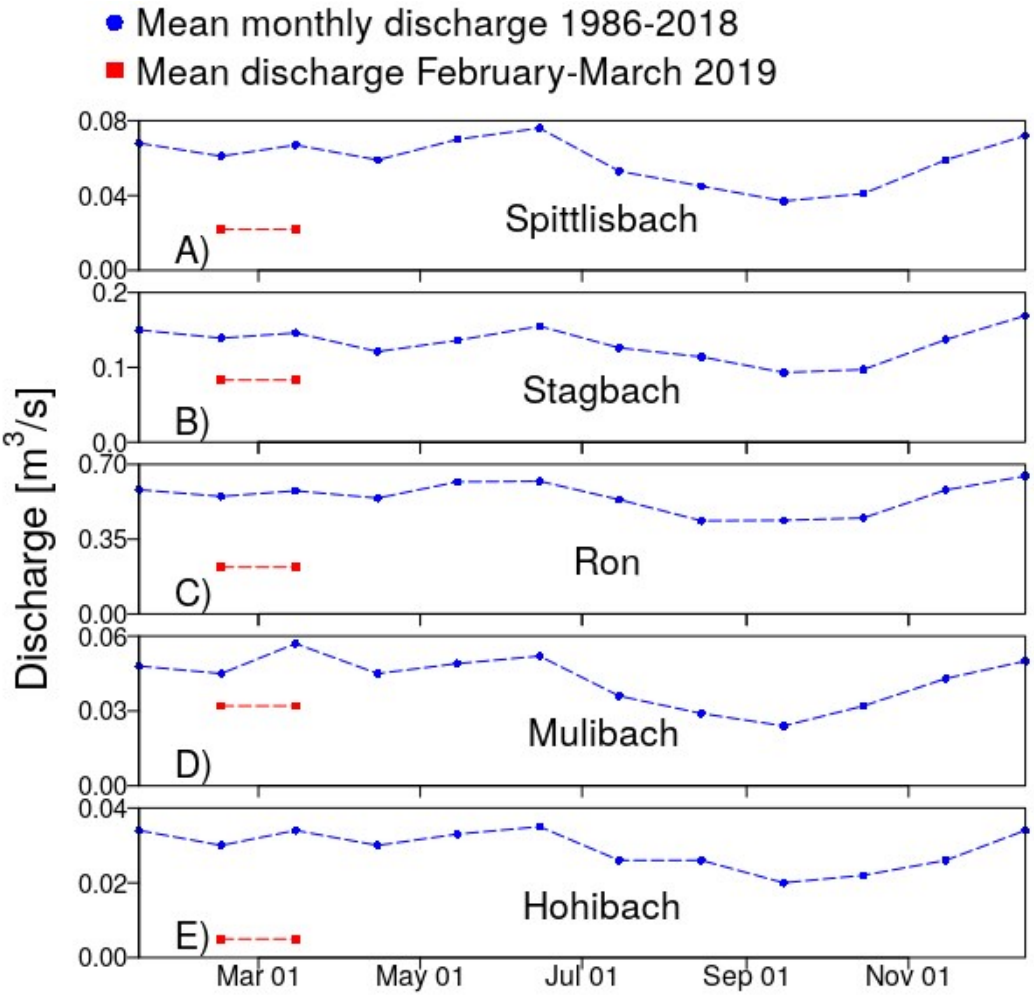

Figure S1: Mean monthly discharge from 1986 to 2018 (blue circles) and mean discharge in February and March 2019 (red squares) in Lake Baldegg tributaries Spittlisbach (panel A), Stagbach (panel B), Ron (panel C), Mulibach (panel D), and Hohibach (panel E).[6] Note that scales of y-axes differ among the tributaries.

Table S4: All crops cultivated in Lake Baldegg catchment during the vegetation period 2019 divided according to their fertilization in February or March 2019.

Non-fertilized	Mineral fertilized	Organically/mineral fertilized	Organically fertilized
- extensively used meadows - hedges, field and bank shrubs (with herb seed) - scattering area - protein peas for forage - hedges, field and bank shrubs (with buffer strips) - vegetable crops in sheltered cultivation without solid foundations (polytunnel) - arable crop protection strip - fruit plants (pears) - fruit plants (stone fruit) - perennial berries - extensively used pastures - hemp - other fruit plants - rotational breach - vineyards with natural biodiversity - broad beans for forage - fallow land - riparian meadow (without pastures) along watercourses	- fruit plantations - Christmas trees - annual berries - seed potato - vineyards - horticultural cultures in sheltered cultivation without solid foundations (polytunnel) - asparagus - other fruit plants - outdoor vegetables for cans - other crops in protected cultivation without solid foundations, subsidized	- annual outdoor vegetables - annual horticultural crops - sugar beet - potatoes	- artificial meadow - other permanent meadows - pastures - silage and green maize - spelt - einkorn wheat - winter oilseed rape - winter barley - winter wheat - grain maize - triticale - mix of broad beans, protein peas and lupines for forage with wheat - winter rape as renewable resource - other artificial meadows - other areas within agricultural land use, non-subsidized - other green areas - spring wheat - less intensively used meadows - forage wheat - fodder beet - oats - other arable land, non-subsidized - silage grain - other arable land, subsidized - spring rape for edible oil production

S2 Material and methods

S2.1 Sampling

S2.1.1 Passive sampling

Calibration of Empore™ SDB-RPS disks for 17β-estradiol and estrone in a channel system. Passive samplers using Empore™ SDB-RPS disks as a receiving phase were calibrated in a channel system.[7] The system with a volume of 700 L was continuously replenished with a mix of 1) ambient water from the river Chriesbach (15.4 L/h) and 2) Chriesbach water passed over a cation exchange resin (5.7 L/h). A reduction of river water hardness was implemented to reduce scaling within the system. Furthermore, Chriesbach water passed by a sediment trap to reduce input of suspended particulate matter into the system; the Chriesbach carries high suspended particulate matter loads during rain events.

Basic parameters: flow rate, temperature and pH. Water flow rate, measured in proximity of the samplers using a MiniAir2 (Schiltknecht, Gossau, Switzerland), was 0.14 ± 0.006 m/s. This value is typical of Swiss midland rivers such as those of the Lake Baldegg catchment. Water temperature was recorded with data loggers (Onset UA-002-08 HOBO 8K, Bakrona, Zürich, Switzerland) at 15 min intervals and the mean temperature (± standard deviation) was 14.9 ± 0.3 °C. pH was recorded with a pHD-S sc probe (15 min interval, Hach Lange, Rheineck, Switzerland). The mean pH was 8.0 ± 0.05.

Peak estrogen concentrations to mimic a runoff event. Water in the channels was continuously spiked with E1 and E2β to a nominal concentration of 20 ng/L. After approximately 6 days in the experiment, the supply of river water was halted and a single dose of spike solution was added to nominal concentrations to 200 ng/L. After 1 h of recirculation, water supply to the channels was restarted again which caused a gradual wash-out of the peak concentration back to a nominal spiking concentration of 20 ng/L. The reason for this spiking scheme was to mimic a peak concentration profile that could occur in the river after slurry application and a subsequent rain event – washing estrogen residues from the fields into the river.

Grab water samples (ca. 600 mL) were taken from the channel system at intervals (Fig. S2) and stored at −18 °C. Following the collection of all water samples, samples were thawed and 400 mL of water were filtered, spiked with internal standards (17β-Estradiol-2,4,16,16-d_4 and estrone-2,4,16,16-d_4) and further processed using solid phase extraction.[8] The extract was evaporated under nitrogen and taken up in 100 µL 50:50 methanol:water for LC-MS/MS.

Sampler conditioning and extraction. Empore™ SDB-RPS disks were conditioned and placed in steel holders.[9] At t = 0 h, 20 RPS disks were placed in the channel, subsequently disks were removed from the channels at intervals (Fig. S2). For disk extraction method, see main text. The final extract was reduced under nitrogen and taken up in 100 µL 50:50 methanol:water for LC-MS/MS. Extracts from water and from passive samplers were analysed by LC-MS/MS as described earlier.[8]

Modelling of passive sampler uptake data. Passive sampler uptake data for E2β and E1 are shown in Fig. S2. Using ModelMaker (Version 4.0), data on water concentrations (C_w, µg/L) and sampler concentration (C_s, µg/kg) were fitted to the differential equation (Eq.1):

$$(1)\ \frac{dC}{dt} = k_{ws} \times C_w - k_{sw} \times C_s$$

This resulted in fitted uptake (k_{ws}, L/kg,d) and release rate (k_{sw}, 1/d) constants. k_{ws} multiplied by the sampler mass (0.332 g) equals the instantaneous sampling rate at t=0, R_{S_t0}.

Passive sampling Lake Baldegg tributary Ron. All passive sampling sites along the main Lake Baldegg tributary Ron are listed in Table S5.

Modelled five days field sampling rate – R_{S_5d}. Both k_{ws} and k_{sw} were used to model uptake under a scenario with a constant concentration of 1 ng/L over 5 days (see also Vermeirssen et al.[10] and Fernandez et al.[11]). A five day sampling window was chosen to match field deployments. Uptake at day 5 was determined (3.4 ng per SDB-disk for E1 and 3.3 ng per SDB-disk for E2β, Fig. S2) and divided by 5 (1 ng/L times 5 days) to obtain a 5 day sampling rate (R_{S_5d}) of 0.66 L/d for E2β and 0.68 L/d for E1.

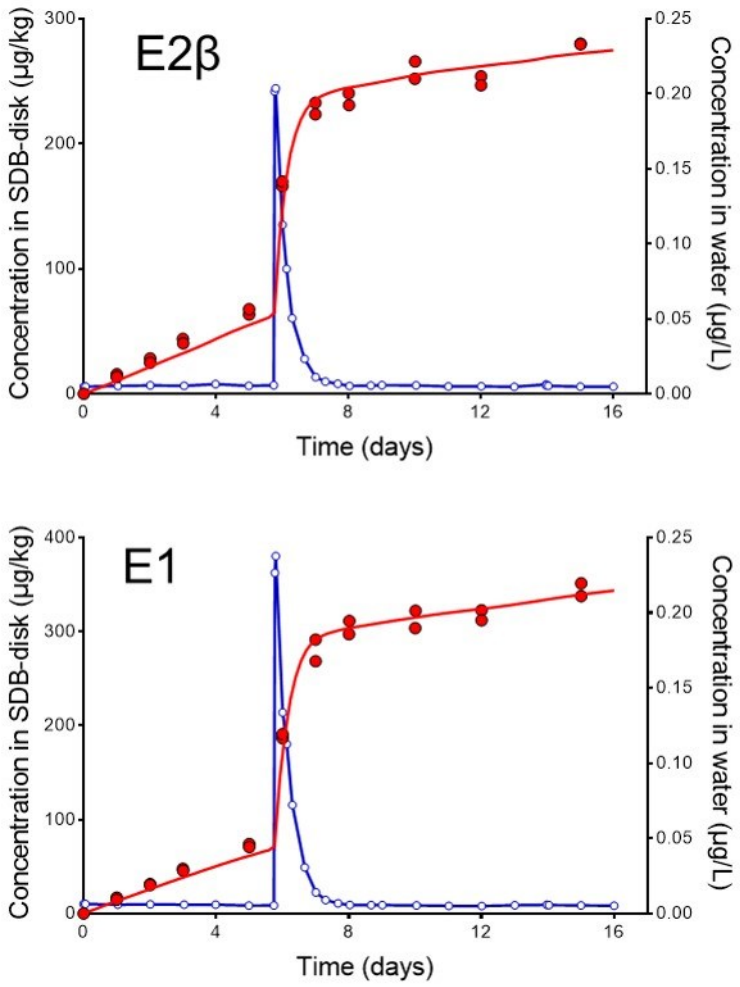

Figure S2: Top 17β-estradiol (E2β), bottom estrone (E1). Blue circles: water sampling interval and aqueous concentration (µg/L). Red circles: concentration per SDB-disk (µg/kg). Lines are model fits.

Table S5: Coordinates (Coordinate system CH1903+ / LV95 (EPSG: 2056)) of the passive sampling sites along Lake Baldegg tributary Ron, Switzerland (for location on the map, see Fig. 1).

Sampling sites passive sampling	Coordinates [Y/X]
Ohmelinge, Hildesrieden	2660489/1222017
Gundolinge, Rain	2661439/1221062
Urswil, Hochdorf	2664749/1222173
Ronfeld, Hochdorf	2664039/1224128

S2.1.2 Time-proportional sampling

Table S6 presents the exact location of the time-proportional water samplers in Lake Baldegg tributaries.

Table S6: Coordinates (Coordinate system CH1903+ / LV95 (EPSG:2056)) and altitude of time-proportional water sampling sites with water gauge and limnograph in Lake Baldegg tributaries Spittlisbach, Stagbach, Ron, Mulibach, and Hohibach (for location on the map, see Fig. 1).[6]

Sampling sites time-proportional water sampling	Coordinates [Y/X]	Altitude [m a.s.l.]
Spittlisbach	2000663/1000226	474
Stagbach	2000663/1000226	466
Ron	2000663/1000224	468
Mulilbach	2000661/1000227	476
Hohibach	2000663/1000227	479

The time-proportional water sampler (Automated Liquid Samper QS300, Quality Environment Limited, Cheltenham, England) is only mechanically enforced. No additional energy is needed for sampling (Fig. S3). The water sampler chamber (sampler chamber volume is 3 L, Fig. S3A) is placed under water at constant depth to obtain a constant sampling rate (Fig. S3B). The regulator (white part, Fig. S3A) determines the sampling rate. The regulator controls the rate at which stream water substitutes the released air from the chamber. The water is forced through an opening (6 mm diameter) with hydrostatic pressure forces.

Every time-proportional water sampling site was equipped with a pressure sensor to measure the water gauge. The minimal, mean and maximal daily discharge was calculated based on the water gauge and the cross-sectional area of the river.

A)  B) 

Figure S3: Time-proportional water sampler releases air, which is replaced by inflowing stream water, at controlled rate (panel A). The sampling device is installed at constant depth in the stream (panel B; for location on the map, see Fig. 1).

S2.2 Chemical analyses

S2.2.1 Chemicals

Dansyl chloride (≥ 99.0% purity) and sodium carbonate (≥ 99.5% purity) were purchased from Sigma-Aldrich (Buchs, Switzerland). Formic acid, which was obtained from VWR chemicals (Dietikon, Switzerland), was used as modifier. Sodium chloride (GR for analysis), water for chromatography (LC-MS grade), potassium persulfate, sulfuric acid, sulfamic acid, ascorbic acid and phosphate standard solution 1000 mg PO_4/L were acquired from Merck (Buchs, Switzerland). Potassium antimony(III) tartrate hydrate was produced by Fluka/Honeywell (Volketswil, Switzerland). All solvents, i.e., acetonitrile, acetone, methanol, water, and MTBE, were of HPLC grade or better. Solvents were bought from either Merck, VWR chemicals, Sigma-Aldrich or Honeywell/Fluka.

S2.2.2 Natural estrogens: method validation

Some target analytes and the corresponding deuterated internal standards interfered with matrix in surface water samples. We had deuterated internal standards for all targeted substances (E2α, E2β, E1, and E3) to compensate for matrix effects. After derivatization of both extracted analytes and deuterated internal standards, the ratio of the analyte and

deuterated internal standard signals of the surface water sample was calibrated against the ratio of derivatized analyte and internal standard signals in pure solvent.

Ion suppression was derived by comparing signals of analytes, which were first dissolved in the final extract of a surface water sample (i.e., matrix matched calibrations) and then derivatized with the derivatized analytes in pure solvent. Injection volume was identical for derivatized analytes in pure solvent and in matrices. By definition, the ion suppression equals 1 minus the ratio of the extacted curve's slope and the slope of the curve for the pure solvent.

For four different analyte concentrations (0 ng/L, 10 ng/L, 20 ng/L, 30 ng/L) a triplicate of samples was produced (equals n=12). The absolute recovery of natural estrogen extraction from surface water was determined by calculating the ratio of the slope of the surface water samples to which the analytes were added before extraction and the internal standard after extraction and the slope of the matrix matched calibration. Relative recovery of natural estrogen extraction from surface water was derived by dividing the slope of the samples to which the analyte and the internal standard were spiked to the surface water samples before extraction by the slope of the matrix matched calibration.

Five replicates of a surface water sample were extracted to appraise method precision. Instrument precision was evaluated by measuring one extracted surface water sample five times.

We derived limit of detection (LOD) and limit of quantification (LOQ) as described in Keith et al..[12] The standard deviation of the mean signal response of a surface water sample was multiplied by 3 to obtain LOD and by 10 to get LOQ.

Table S7: Quality assurance and quality control parameters for natural estrogen extraction from surface waters: ion suppression (negative values indicate enhancement), absolute and relative recoveries, method and instrument precisions, limits of detection (LOD) and limit of quantification (LOQ). The letter n refers to the number of samples per concentration and c indicates number of different concentrations assessed.

Surface water	17α-estradiol (E2α)	17β-estradiol (E2β)	Estrone (E1)	Estriol (E3)
Ion suppression [%] *(n=3, c=4)*	−3	2	−6	−1
Absolute recovery [%] *(n=3, c=4)*	99	96	122	100
Relative recovery [%] *(n=3, c=4)*	125	100	109	100
Method precision [%] *(n=5, c=1)*	8	4	2	5
Instrument precision [%] *(n=5, c=1)*	6	3	2	4
LOD [ng/L]	0.11	0.10	0.07	0.08
LOQ [ng/L]	0.36	0.34	0.24	0.25

S2.2.3 Natural estrogens: stability and contamination experiments time-proportional sampling

Water samples were collected time-proportionally over 24 hours with a sampling device immersed in stream water. Our goal was to study estrogen degradation in the sampler during sampling (Fig. S3B). We simulated the fate of an estrogen molecule in the sampler by placing an aluminum bottle containing surface water spiked with natural estrogens (resulting concentration 5 ng/L) for 24 hours in a tributary. The natural estrogen concentrations were determined in the water filled to the aluminum bottle before spiking, directly after spiking and after 24 hours in the stream. We conducted this experiment in three different tributaries on the same day. The simulated storage in the sampler resulted for all natural estrogens in concentration changes in the range of method precision.

Some samples were stored at −20°C prior to analysis. Therefore, estrogen stability during storage was tested. Surface water was spiked with natural estrogens (resulting concentration 5 ng/L) and divided in subsamples. The subsamples were frozen at −20°C in aluminum bottles (300 mL). Natural estrogen concentrations in the subsamples were determined after 24 hours, 48 hours, 7 days and 30 days. As shown in Fig. S4, we did not observe a major degradation of the compounds during storage at −20°C.

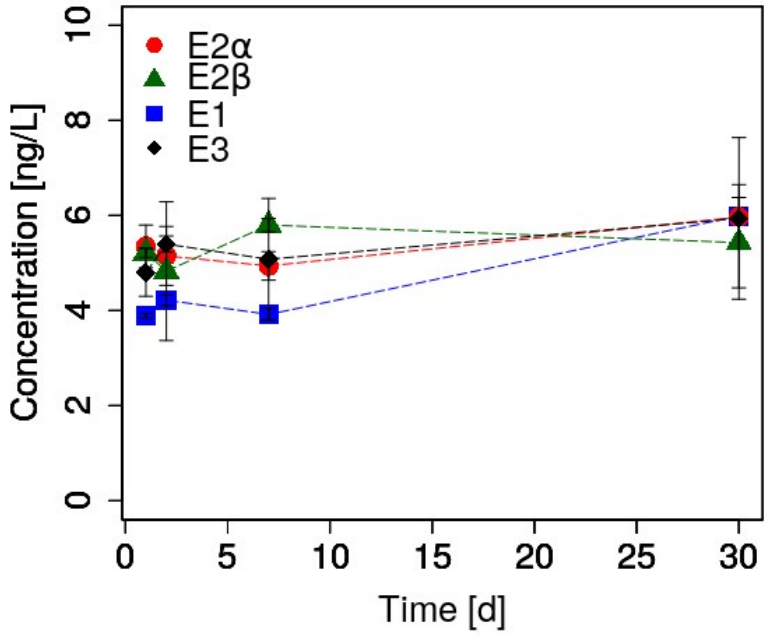

Figure S4: Natural estrogen concentrations in subsamples of surface water spiked (5 ng/L) with 17α-estradiol (E2α, red circles), 17β-estradiol (E2β, green triangles), estrone (E1, blue squares), and estriol (E3, black diamonds) after 24 hours, 48 hours, 7 days, and 30 days of storage at −20°C. All natural estrogen concentrations remained relatively stable during storage. Error bars show standard deviations of triplicates of samples.

A sample of Milli-Q water (Milli-Q Gradient, Merck, Darmstadt, Germany) was transported to the field and back to the laboratory. Thereby, we assessed field blank contamination. No contamination occurred during the transport.

Possible background contamination of the sampling device was studied with a sampler blank. In our study, we rinsed three sampling devices three times with Milli-Q water to obtain sampler blanks. Natural estrogen concentrations in all sampler blanks were <LOQ.

Furthermore, we investigated stability of natural estrogens during transport from the field to the laboratory. For all tributaries, a water sample was divided into two subsamples. The subsamples were spiked in the field and in the laboratory, respectively. Likewise, we verified possible degradation processes during transport. We conducted this experiment for every monitored tributary on the same experimental day. It was observed that E1 was increased by 9% when comparing samples spiked in the field to samples spiked in the laboratory, while E2α was decreased by 9%, E2β by 7%, and E3 by 8%.

Contamination during extraction was tested with a blank in every extraction batch. We used Milli-Q water as blank. A blank contamination of 0.25 ng/L was registered on February 25, 2019. No other blank was contaminated.

S2.2.4 Phosphorus

Potassium persulfate solution (10 mL) was added to 50 mL of the water sample to determine total phosphorus. Afterward, the sample was autoclaved for 30 min at 120 °C (Systec, Tuttnauer, Breda, The Netherlands). Thereby, orthophosphate was formed. For the determination of dissolved phosphorus the sample was filtered before autoclavation. The autoclaved solution was filtered (45 membran filter, 0.45µm). Molybdate sulfuric acid reagent (2 mL) and 1 mL of ascorbic acid solution was added to the non-filtered (total phosphorus concentration in water) and filtered (dissolved phosphorus concentration in water) autoclaved solutions and to water (orthophosphate concentration in water). Orthophosphate forms in acidic solution a complex with molybdate. Ascorbic acid reduces this complex to phosphorus molybdenum blue. Subsequently, orthophosphate concentrations were quantified with a photometer (Cary 50, Varian, Palo Alto, USA).

S2.3 Biological analysis using ERα-CALUX

Screening of native water samples for estrogenic activity with ERα-CALUX. Water samples were analyzed directly (i.e., without any sample manipulation, such as filtration or extraction) for their estrogenic activity in the *in vitro* ERα-CALUX bioassay.[13, 14] ERα-CALUX determines

total estrogenic activity of a sample containing a chemical mixture of unknown composition. Estrogenic chemicals can bind to the estrogen receptor and induce a transcriptional cascade resulting in a production of the luciferase enzyme. This enzyme degrades the substrate, luciferin present in the assay medium and produces a quantifiable luminescence light in a proportional manner.

The ISO 19040:3 protocol was followed when analyzing the native surface water samples with the ERα-CALUX.[15] Serial, two-fold dilutions of each Lake Baldegg tributary sample (1-2-4x of the 138 surface water samples), five dilutions of the spiked nanopure water and spiked surface water samples (randomly selected), and ten dilutions of the reference chemical, i.e. E2β, were tested in duplicate in the screening. Lake Baldegg tributary samples showing an estrogenic activity (>LOQ of 0.50 ng EEQ/L) in the screening were repeated and tested in more dilutions together with the reference, i.e. E2β, both in triplicate. Each measured response (relative luminescence light unit) per test concentration was then normalized based on the negative control (nanopure water, which was also used to prepare the sample dilutions) as 0% and the maximum fitted response of the reference curve (E2β) as 100%. An effect level of 10% was set as a quantification level. The sample dilution reaching the 10% effect level was interpolated from the normalized reference curve and E2β equivalent concentration (EEQ_{bio}) was determined after correcting it for the respective sample dilution.[16]

Characterization of quantification limits in native water samples and method validation. To characterize what level of estrogenic activity could be quantified at the 10% effect level set in native water samples, first nanopure water (representing no sample matrix) was spiked with the reference chemical, E2β at different concentrations ranging from 4.5 down to 0 ng E2β/L and tested multiple times (n=4, Fig. S5).

This experiment revealed a quantification limit of 0.50 ng EEQ_{bio}/L in the spiked nanopure samples. This was the concentration level where 10% estrogenic effect was induced. It is in accordance with the quantification limit range of 0.30 – 1.0 ng EEQ_{bio}/L stated in the ISO 19040:3 for direct testing of water samples in ERα-CALUX.

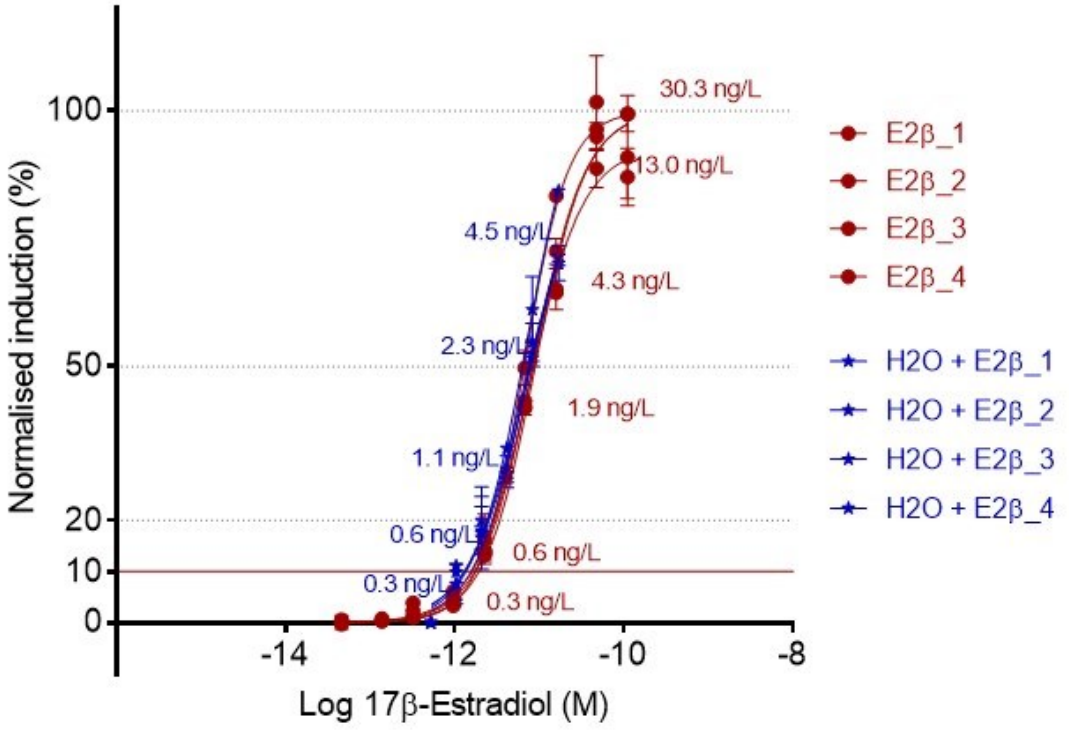

Figure S5: Concentration-response relationships for nanopure water samples spiked with 17β-estradiol (E2β) (H2O + E2β, blue) tested in four independent runs in ERα-CALUX against the E2β reference (tested in each run and on each test plate, red). The logarithmically scaled x-axis shows the E2β molar concentration. On the y-axis, the normalized bioassay response (%) is indicated. Measurement precision was nearly perfect, RSD<5%, when comparing either the 10% (as LOQ) or the 50% (when applicable) effect levels of the spiked samples with those levels of the respective reference, E2β.

As a next step, a set of native surface water samples from Lake Baldegg tributaries (representing the true sample matrix) were spiked with E2β the same as the nanopure waters to validate the *in vitro* estrogenicity screening of native water samples. Eight spiked samples were tested in two independent runs in duplicates (serving as biological replicates) and another six spiked samples in a single run in duplicates (technical replicates, Fig. S6). All spiked samples showed excellent measurement precision (<5%) and negligible differences (relative error) between the measured (in the spiked sample) and the true value measured for the reference, E2β (i.e., EC_{10} or EC_{50}; effect concentrations inducing 10% or 50% effect levels).

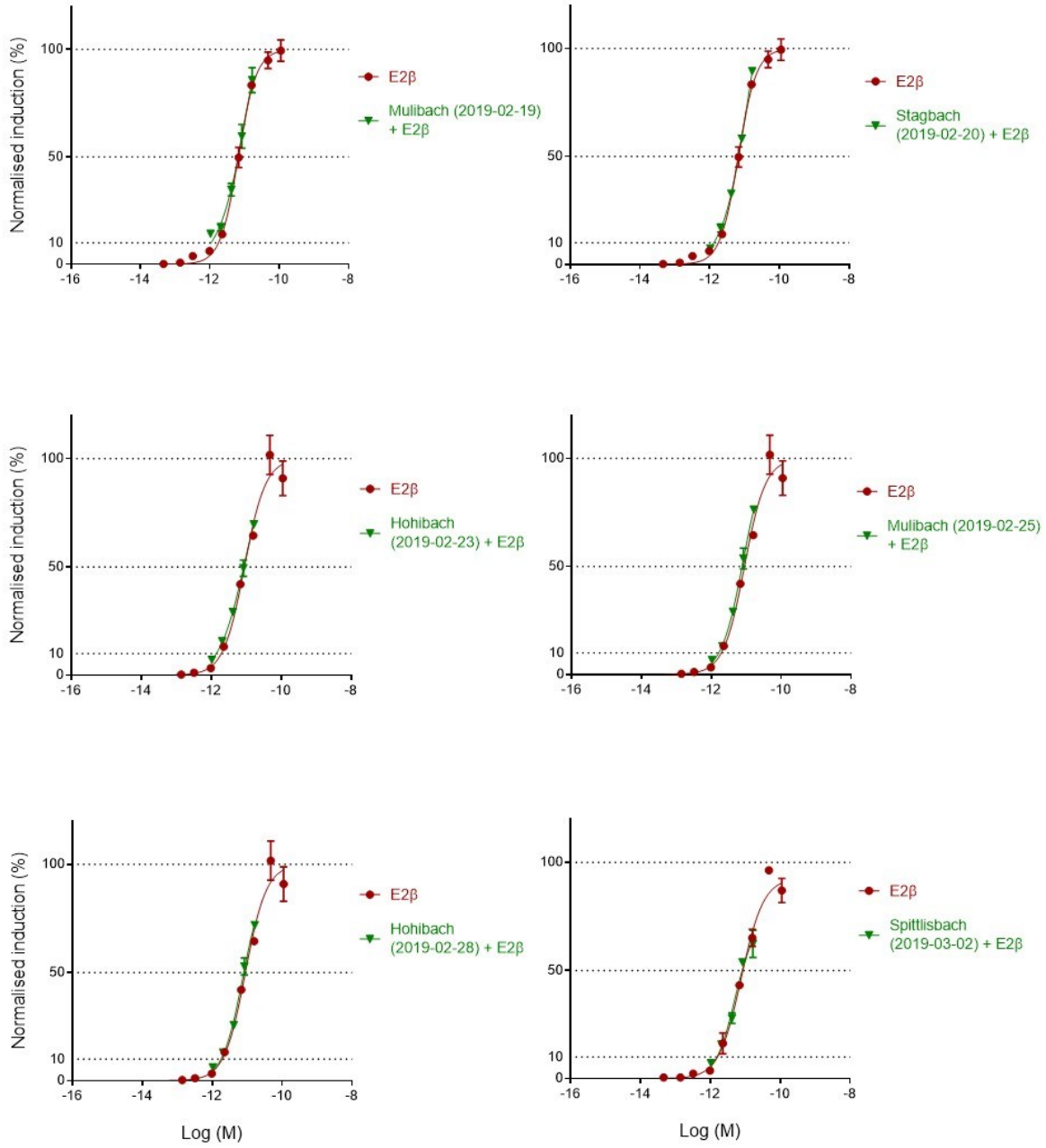

Figure S6: Representative concentration-response relationships of six Lake Baldegg tributaries water samples spiked with a 17β-estradiol stock solution at a concentration range covered by the reference curve. The curves show nearly perfect overlap between the response given by the reference E2β (red points) and the reference spiked to various native water samples (green triangles). The curves also indicate that no background estrogenic activity was measured in the native water samples, otherwise the sample curves would have shifted up vertically (i.e., inducing higher, additive response at the nominal concentration level).

Relative potency of natural estrogens to E2β – used for chemical EEQ estimation. Measured chemical concentrations of the individual estrogenic chemicals can be translated into and expressed as expected/calculated total estrogenic effect (EEQ_{chem}). To this, individual

concentrations are multiplied with (corrected for) their individual relative potency (defined in the bioassay, Table S8) and summed up. Doing so, chemically measured concentrations can be linked to and directly compared with the measured biological effects (EEQ_{bio}) (Table S12). The relative potencies that we used were either reported earlier (Table S8) or determined in this study (Fig. S7).

Table S8: Overview of the relative potencies of estrogenic chemicals used to estimate the total estrogenic activity of the estrogens measured in the water samples (EEQ_{chem}). An estrogenic chemical with a relative potency of 0.1 is 10 times less potent than E2β (of which relative potency is 1). This means a 10 times higher concentration is needed to induce the same effect as E2β.

Relative potency to E2β	**Könemann et al., 2018[17]**	**This study**
17α-estradiol (E2α)	-	0.008
17β-estradiol (E2β)	1	-
estrone (E1)	0.01	-
estriol (E3)	-	0.086

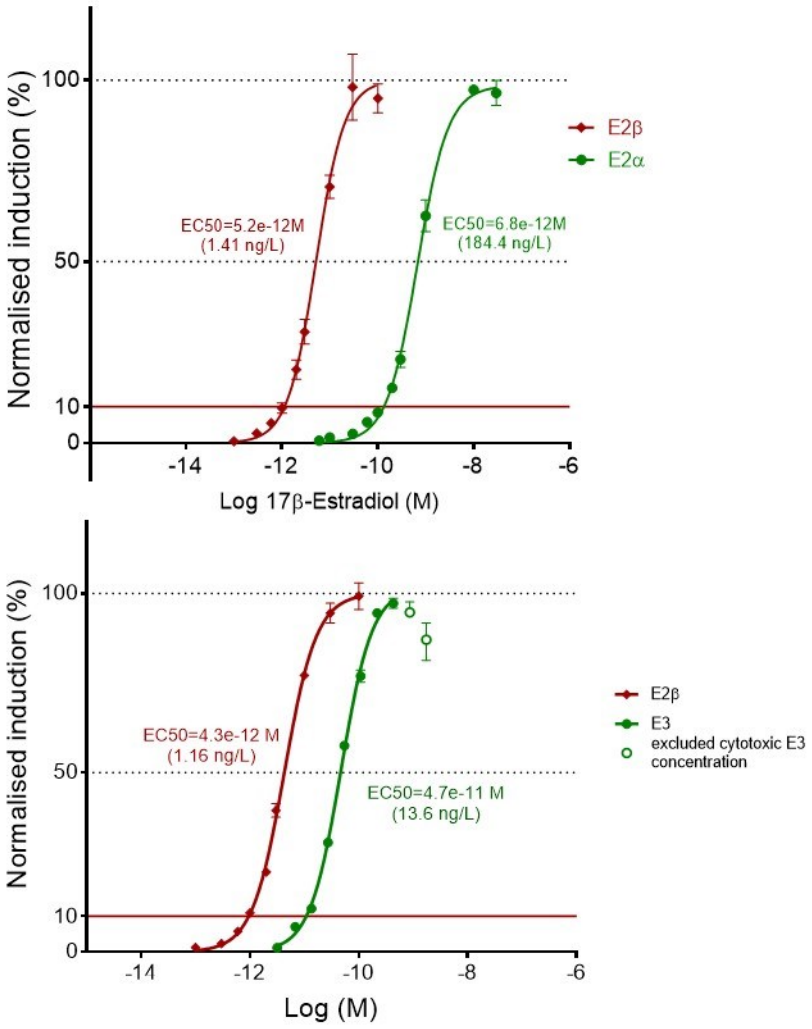

Figure S7: Concentration-response relationships for 17α-estradiol (E2α) (upper panel), estriol (E3) (lower panel) and the reference substance, 17β-estradiol (E2β) in ERα-CALUX. Relative potency of E2α and E3 towards E2β was defined based on their 50% effect level (EC_{50}): $EC_{50\ E2\beta} / EC_{50\ E3\ or\ E2\alpha}$.

S2.4 Estimation of natural estrogen concentrations in tributaries including an uncertainty assessment

In a nationwide monitoring natural estrogen concentrations in slurry (c_{slurry}) (Table S9) were determined.[18] We monitored cattle (n=17) and pig (n=9) slurry pits in Switzerland. For the Monte Carlo simulation, we used a log-normal distribution with mean and standard deviations of natural estrogen concentrations in cattle ($c_{slurry,cattle}$) and pig ($c_{slurry,pig}$) slurry pits (Table S9). Values below LOQ were set to LOD and then logarithmised.

Table S9: Mean (μ) and standard deviations (σ) of 17α-estradiol (E2α), 17β-estradiol (E2β), estrone (E1) and estriol (E3) concentrations (c_{slurry}) in Swiss cattle ($c_{slurry,cattle}$, n=17) and pig ($c_{slurry,pig}$, n=9) slurry pits.[18]

c_{slurry}	E2α [ng/L] μ±σ	E2β [ng/L] μ±σ	E1 [ng/L] μ±σ	E3 [ng/L] μ±σ
Cattle slurry ($c_{slurry,cattle}$, n=17)	861±367	138±126	160±206	397±411
Pig slurry ($c_{slurry,pig}$, n=9)	70±108	54±105	160±210	244±406

On a tile-drained test field we applied cattle (n=4) and pig (n=5) slurry, collected drainage water samples from the tile drain flow proportionally and determined natural estrogen concentrations in slurry and drainage water. Subsequently, the fraction of natural estrogens in slurry emitted to drainage water was derived (E_F). Minimal ($E_{F,min}$) and maximal ($E_{F,max}$) emitted fractions of natural estrogens in cattle ($E_{F,cattle}$) and pig ($E_{F,pig}$) slurries are shown in Table S10.[19] A uniform distribution with minimal and maximal emitted fractions of natural estrogens in cattle and pig slurries was used for the Monte Carlo simulation. Gall et al.[20] used a comparable experimental set up in the United States and obtained similar emitted fractions.

Table S10: Minimal (min) and maximal (max) emitted fractions (E_F) of slurry-derived 17α-estradiol (E2α), 17β-estradiol (E2β), estrone (E1), and estriol (E3) to drainage water of a tile-drained agricultural field. Cattle ($E_{F,cattle}$, n=4) and pig ($E_{F,pig}$, n=5) slurries were applied on a tile-drained agricultural field to determine the emitted fractions of natural estrogens to surface water.[19]

E_F	E2α [%] ($E_{F,min}$-$E_{F,max}$)	E2β [%] ($E_{F,min}$-$E_{F,max}$)	E1 [%] ($E_{F,min}$-$E_{F,max}$)	E3 [%] ($E_{F,min}$-$E_{F,max}$)	Etot ($E_{F,min}$-$E_{F,max}$)
Cattle slurry ($E_{F,cattle}$, n=4)	0.02-0.47	0.00-0.31	0.06-0.89	0.01-0.85	
Pig slurry ($E_{F,pig}$, n=5)	0.00-0.23	0.00-0.06	0.00-1.46	0.00-0.69	
Gall et al.[20]					0.23-0.37

All parameter values containing uncertainties and associated statistical distributions used to estimate mean natural estrogen concentrations in Lake Baldegg tributaries are listed in Table S11.

Table S11: Parameter values with uncertainties and associated statistical distributions, used to estimate mean natural estrogen concentrations in Lake Baldegg tributaries (c_{water}) from February to March 2019. Normal distribution is indicated by N(μ,σ) with mean μ and standard deviation σ. Uniform distribution between a and b is represented by Unif(a,b). Parameters which are log-normal distributed are shown with logN(μ,σ) with mean μ and standard deviation σ.

Parameter	Abbreviation	Unit	Distribution	Source
Annual volume of slurry produced per cattle/pig	V_{slurry}	L	$N(\mu_{V,slurry}, \mu_{V,slurry} \times 0.1)$	Richner et al.[21]
Natural estrogen concentration in slurry	c_{slurry}	ng/L	$logN(\mu_{c,slurry}, \sigma_{c,slurry})$	Table S9
Number of slurry applications per year	A_{slurry}		*Unif*(2, 5)	Richner et al.[21]
Emitted fraction of natural estrogens to surface waters	E_F	%	$Unif(E_{F,min}, E_{F,max})$	Table S10, Gall et al.[20]

We tested the response of the mean natural estrogen concentration estimations with a sensitivity analysis on the parameters of the Monte Carlo simulation. In the sensitivity analysis, the standard deviation of every input parameter was set to 10% of its mean. The resulting change in standard deviation relative to the mean estimated natural estrogen concentrations in Lake Baldegg tributaries was calculated. For parameters with a uniform distribution, the normal distribution with a standard distribution of 10% of the mean was used in the sensitivity analysis (Table S16).

S3 Results

S3.1 Natural estrogen occurrence as determined by time-proportional water sampling

Table S12 shows daily individual natural estrogen concentrations, chemical and biological EEQ concentrations in Lake Baldegg tributaries from February 17 to March 18, 2019.

Table S12: Daily concentrations of 17α-estradiol (E2α), 17β-estradiol (E2β), estrone (E1), and estriol (E3) in Lake Baldegg tributaries Ron, Mulibach, Hohibach, Spittlisbach, and Stagbach from February 17 to March 18, 2019. Concentrations below limit of detection (LOD) were zeroed and concentrations between LOD and limit of quantification (LOQ) were set to LOD. Chemical 17β-estradiol equivalent concentration (EEQ_{chem}) was calculated based on the determined concentrations of E2α, E2β, E1, and E3 in tributaries during the sampling period. Biological EEQ (EEQ_{bio}) was determined with ERα-CALUX except for a few samples (-). For native water samples in ERα-CALUX the LOQ was 0.50 ng EEQ/L.

Sampling Date	Tributary	E2α [ng/L]	E2β [ng/L]	E1 [ng/L]	E3 [ng/L]	EEQ_{chem} [ng/L]	EEQ_{bio} [ng/L]
2019-02-17	Ron	0.67	0.10	0.07	0.08	0.11	<LOQ
2019-02-17	Mulibach	0.00	0.00	0.00	0.00	0.00	-
2019-02-17	Hohibach	0.57	0.10	0.00	0.08	0.11	<LOQ
2019-02-17	Spittlisbach	0.44	0.10	0.07	0.08	0.11	<LOQ
2019-02-17	Stagbach	0.44	0.10	0.00	0.08	0.11	<LOQ
2019-02-18	Ron	0.38	0.10	0.07	0.08	0.11	<LOQ
2019-02-18	Mulibach	0.42	0.10	0.07	0.00	0.10	<LOQ
2019-02-18	Hohibach	0.11	0.00	0.07	0.00	0.00	<LOQ
2019-02-18	Spittlisbach	0.60	0.00	0.30	0.00	0.01	<LOQ
2019-02-18	Stagbach	0.55	0.10	0.07	0.08	0.11	<LOQ
2019-02-19	Ron	0.11	0.00	0.07	0.00	0.00	<LOQ
2019-02-19	Mulibach	0.11	0.00	0.07	0.00	0.00	<LOQ
2019-02-19	Hohibach	0.11	0.00	0.00	0.00	0.00	<LOQ
2019-02-19	Spittlisbach	10.5	0.00	2.5	0.67	0.17	0.72
2019-02-19	Stagbach	0.11	0.00	0.25	0.00	0.00	<LOQ
2019-02-20	Ron	0.44	0.00	0.00	0.80	0.07	<LOQ
2019-02-20	Mulibach	0.50	0.10	0.00	0.71	0.16	<LOQ
2019-02-20	Hohibach	0.53	0.00	0.33	0.97	0.09	<LOQ
2019-02-20	Spittlisbach	0.44	0.10	0.07	0.67	0.16	<LOQ
2019-02-20	Stagbach	0.51	0.00	0.32	0.62	0.06	<LOQ
2019-02-21	Ron	0.38	0.00	0.00	0.08	0.01	<LOQ
2019-02-21	Mulibach	0.11	0.00	0.00	0.00	0.00	<LOQ
2019-02-21	Hohibach	0.11	0.00	0.00	0.08	0.01	<LOQ
2019-02-21	Spittlisbach	0.11	0.00	0.00	0.00	0.00	-
2019-02-21	Stagbach	0.11	0.61	3.5	0.99	0.73	<LOQ
2019-02-22	Ron	0.11	0.10	0.07	0.08	0.11	<LOQ
2019-02-22	Mulibach	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-02-22	Hohibach	0.11	0.00	0.00	0.00	0.00	<LOQ
2019-02-22	Spittlisbach	0.11	0.10	1.0	0.08	0.12	<LOQ
2019-02-22	Stagbach	0.11	0.00	0.95	0.08	0.02	<LOQ
2019-02-23	Ron	0.11	0.00	0.07	0.59	0.05	<LOQ
2019-02-23	Mulibach	0.11	0.00	0.00	0.00	0.00	<LOQ
2019-02-23	Hohibach	0.11	0.00	0.07	0.00	0.00	<LOQ
2019-02-23	Spittlisbach	0.11	0.00	0.00	0.00	0.00	<LOQ
2019-02-23	Stagbach	0.11	0.00	0.07	0.00	0.00	<LOQ
2019-02-24	Ron	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-02-24	Mulibach	0.11	0.00	0.00	0.00	0.00	<LOQ
2019-02-24	Hohibach	0.11	0.00	0.00	0.00	0.00	<LOQ

2019-02-24	Spittlisbach	0.11	0.00	0.00	0.00	0.00	<LOQ
2019-02-24	Stagbach	0.11	0.00	0.07	0.00	0.00	<LOQ
2019-02-25	Ron	0.46	0.00	0.07	0.47	0.05	<LOQ
2019-02-25	Mulibach	0.00	0.00	0.07	0.08	0.01	<LOQ
2019-02-25	Hohibach	0.00	0.00	0.00	0.00	0.00	<LOQ
2019-02-25	Spittlisbach	0.00	0.00	0.00	0.00	0.00	<LOQ
2019-02-25	Stagbach	0.00	0.00	0.07	0.00	0.00	<LOQ
2019-02-26	Ron	1.2	0.10	0.07	0.08	0.12	<LOQ
2019-02-26	Mulibach	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-02-26	Hohibach	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-02-26	Spittlisbach	0.11	0.00	0.07	0.46	0.04	<LOQ
2019-02-26	Stagbach	0.11	0.00	0.46	0.08	0.01	<LOQ
2019-02-27	Ron	0.37	0.00	0.07	0.08	0.01	<LOQ
2019-02-27	Mulibach	0.00	0.00	0.07	0.08	0.01	<LOQ
2019-02-27	Hohibach	0.11	0.00	0.07	0.00	0.00	<LOQ
2019-02-27	Spittlisbach	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-02-27	Stagbach	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-02-28	Ron	0.11	0.00	0.46	0.08	0.01	<LOQ
2019-02-28	Mulibach	0.11	0.00	0.00	0.08	0.01	<LOQ
2019-02-28	Hohibach	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-02-28	Spittlisbach	0.11	0.00	0.00	0.00	0.00	<LOQ
2019-02-28	Stagbach	0.11	0.00	0.39	0.08	0.01	<LOQ
2019-03-01	Ron	0.50	0.00	0.72	0.08	0.02	<LOQ
2019-03-01	Mulibach	0.11	0.00	0.56	0.00	0.01	<LOQ
2019-03-01	Hohibach	0.11	0.00	0.61	0.00	0.01	<LOQ
2019-03-01	Spittlisbach	0.41	0.00	0.62	0.00	0.01	<LOQ
2019-03-01	Stagbach	0.11	0.00	0.88	0.08	0.02	<LOQ
2019-03-02	Ron	0.11	0.00	0.07	0.00	0.00	<LOQ
2019-03-02	Mulibach	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-03-02	Hohibach	0.11	0.00	0.07	0.00	0.00	<LOQ
2019-03-02	Spittlisbach	0.11	0.00	1.2	0.08	0.02	<LOQ
2019-03-02	Stagbach	0.00	0.00	1.1	0.00	0.01	<LOQ
2019-03-03	Ron	0.11	0.00	0.49	0.08	0.01	<LOQ
2019-03-03	Mulibach	0.11	0.00	0.30	0.00	0.00	<LOQ
2019-03-03	Hohibach	0.11	0.00	0.07	0.00	0.00	<LOQ
2019-03-03	Spittlisbach	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-03-03	Stagbach	0.11	0.00	0.37	0.00	0.00	<LOQ
2019-03-04	Ron	0.11	0.10	0.00	0.73	0.16	<LOQ
2019-03-04	Mulibach	2.6	0.10	1.5	0.46	0.18	<LOQ
2019-03-04	Hohibach	0.59	0.10	0.00	0.00	0.10	<LOQ
2019-03-04	Spittlisbach	0.11	0.10	0.00	0.45	0.14	<LOQ
2019-03-04	Stagbach	0.68	0.00	0.57	0.32	0.04	<LOQ
2019-03-05	Ron	0.89	0.39	0.07	0.00	0.40	<LOQ
2019-03-05	Mulibach	0.11	0.10	0.24	2.0	0.27	<LOQ
2019-03-05	Hohibach	0.56	0.10	0.07	0.00	0.11	<LOQ
2019-03-05	Spittlisbach	0.37	0.10	0.00	0.78	0.17	<LOQ
2019-03-05	Stagbach	0.00	0.39	0.07	1.3	0.50	<LOQ
2019-03-06	Ron	0.63	0.10	0.00	0.08	0.11	<LOQ

2019-03-06	Mulibach	0.11	0.00	0.34	0.00	0.00	<LOQ
2019-03-06	Hohibach	0.11	0.00	0.00	0.08	0.01	<LOQ
2019-03-06	Spittlisbach	0.11	0.00	0.07	0.26	0.02	<LOQ
2019-03-06	Stagbach	0.11	0.10	0.07	0.58	0.15	<LOQ
2019-03-07	Ron	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-03-07	Mulibach	0.11	0.00	0.00	0.08	0.01	<LOQ
2019-03-07	Hohibach	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-03-07	Spittlisbach	0.45	0.00	0.28	0.08	0.01	0.66
2019-03-07	Stagbach	0.11	0.00	0.27	0.08	0.01	<LOQ
2019-03-08	Ron	0.11	0.10	0.57	0.08	0.11	<LOQ
2019-03-08	Mulibach	0.11	0.10	0.51	0.08	0.11	0.56
2019-03-08	Hohibach	0.11	0.00	0.70	0.31	0.03	<LOQ
2019-03-08	Spittlisbach	0.63	0.10	1.2	0.08	0.12	<LOQ
2019-03-08	Stagbach	0.11	0.10	0.54	0.36	0.14	<LOQ
2019-03-09	Ron	0.11	0.10	0.28	1.2	0.21	<LOQ
2019-03-09	Mulibach	0.11	0.10	0.07	0.08	0.11	<LOQ
2019-03-09	Hohibach	0.11	0.10	0.07	0.08	0.11	<LOQ
2019-03-09	Spittlisbach	0.11	0.10	0.07	0.08	0.11	<LOQ
2019-03-09	Stagbach	0.56	0.00	0.34	1.2	0.11	<LOQ
2019-03-10	Ron	0.52	0.10	0.07	0.36	0.14	<LOQ
2019-03-10	Mulibach	0.45	0.00	0.39	0.08	0.01	<LOQ
2019-03-10	Hohibach	0.11	0.35	0.07	0.32	0.38	<LOQ
2019-03-10	Spittlisbach	0.11	0.10	0.29	0.36	0.13	<LOQ
2019-03-10	Stagbach	0.73	0.10	1.0	0.42	0.15	<LOQ
2019-03-11	Ron	0.11	0.00	0.28	0.30	0.03	<LOQ
2019-03-11	Mulibach	0.11	0.00	0.36	0.34	0.03	<LOQ
2019-03-11	Hohibach	0.11	0.00	0.00	0.27	0.02	<LOQ
2019-03-11	Spittlisbach	0.52	0.00	0.40	0.26	0.03	<LOQ
2019-03-11	Stagbach	0.65	0.10	0.07	0.59	0.16	<LOQ
2019-03-12	Ron	0.11	0.00	0.26	0.25	0.03	2.0
2019-03-12	Mulibach	0.11	0.00	0.36	0.76	0.07	<LOQ
2019-03-12	Hohibach	0.11	0.00	0.07	0.08	0.01	<LOQ
2019-03-12	Spittlisbach	0.00	0.00	0.07	0.25	0.02	<LOQ
2019-03-12	Stagbach	0.00	0.00	0.37	0.08	0.01	<LOQ
2019-03-13	Ron	0.00	0.10	0.07	0.08	0.11	<LOQ
2019-03-13	Mulibach	0.00	0.00	0.43	0.08	0.01	<LOQ
2019-03-13	Hohibach	0.00	0.00	0.07	0.08	0.01	<LOQ
2019-03-13	Spittlisbach	0.00	0.00	0.07	0.00	0.00	<LOQ
2019-03-13	Stagbach	0.11	0.10	0.46	0.08	0.11	<LOQ
2019-03-14	Ron	0.11	0.00	0.47	0.36	0.04	<LOQ
2019-03-14	Mulibach	0.00	0.00	0.25	0.08	0.01	<LOQ
2019-03-14	Hohibach	0.00	0.00	0.07	0.27	0.02	<LOQ
2019-03-14	Spittlisbach	0.00	0.00	0.30	0.08	0.01	-
2019-03-14	Stagbach	0.11	0.00	0.85	0.43	0.05	-
2019-03-15	Ron	0.11	0.43	1.7	1.8	0.60	<LOQ
2019-03-15	Mulibach	0.11	0.00	0.35	0.67	0.06	-
2019-03-15	Hohibach	0.48	0.10	0.73	1.0	0.20	<LOQ
2019-03-15	Spittlisbach	0.11	0.10	0.59	0.43	0.14	-

2019-03-15	Stagbach	0.11	0.00	0.71	0.00	0.01	-
2019-03-16	Ron	0.11	0.00	0.74	0.00	0.01	-
2019-03-16	Mulibach	0.11	0.00	0.51	0.08	0.01	-
2019-03-16	Hohibach	0.11	0.00	0.76	0.00	0.01	-
2019-03-16	Spittlisbach	0.11	0.00	0.70	0.00	0.01	-
2019-03-16	Stagbach	0.11	0.00	0.46	0.00	0.01	-
2019-03-17	Ron	0.11	0.00	0.61	0.08	0.01	0.78
2019-03-17	Mulibach	0.11	0.00	0.67	0.00	0.01	<LOQ
2019-03-17	Hohibach	0.11	0.00	0.58	0.00	0.01	<LOQ
2019-03-17	Spittlisbach	0.11	0.00	0.39	0.00	0.00	<LOQ
2019-03-17	Stagbach	0.11	0.00	1.6	0.00	0.02	<LOQ
2019-03-18	Ron	0.11	0.00	0.54	0.08	0.01	<LOQ
2019-03-18	Mulibach	0.11	0.00	0.61	0.08	0.01	<LOQ
2019-03-18	Hohibach	0.11	0.00	0.73	0.00	0.01	<LOQ
2019-03-18	Spittlisbach	0.11	0.00	0.74	0.00	0.01	<LOQ
2019-03-18	Stagbach	0.11	0.00	0.46	0.00	0.01	<LOQ

The maximal and mean natural estrogen concentrations measured in every tributary from February 17 to March 18, 2019 are listed in Table S13.

Table S13: The maximal and mean concentrations of 17α-estradiol (E2α), 17β-estradiol (E2β), estrone (E1), and estriol (E3) in Lake Baldegg tributaries Spittlisbach, Stagbach, Ron, Mulibach, and Hohibach from February 17 to March 18, 2019. To calculate the mean values, values below LOD were zeroed and values between LOD and LOQ were set to the LOD of the corresponding natural estrogen. The maximal and mean concentrations were calculated based on 30 daily time-proportional samples per catchment.

Tributary	**Maximal concentration [ng/L]**				**Mean concentration [ng/L]**			
	E2α	**E2β**	**E1**	**E3**	**E2α**	**E2β**	**E1**	**E3**
Spittlisbach	10.5	0.10	2.5	0.78	0.54	0.03	0.37	0.18
Stagbach	0.73	0.61	3.5	1.3	0.21	0.06	0.55	0.26
Ron	1.2	0.43	1.7	1.8	0.28	0.06	0.27	0.27
Mulibach	2.6	0.10	1.5	2.0	0.21	0.02	0.28	0.21
Hohibach	0.59	0.35	0.76	1.0	0.17	0.03	0.18	0.13

The daily and the daily cumulative natural estrogen loads exported through the monitored tributaries to Lake Baldegg are shown in Fig. S8 and Fig. S9, respectively. The daily natural estrogen load in each tributary was the daily natural estrogen concentration multiplied by the cumulative discharge volume of the corresponding day.

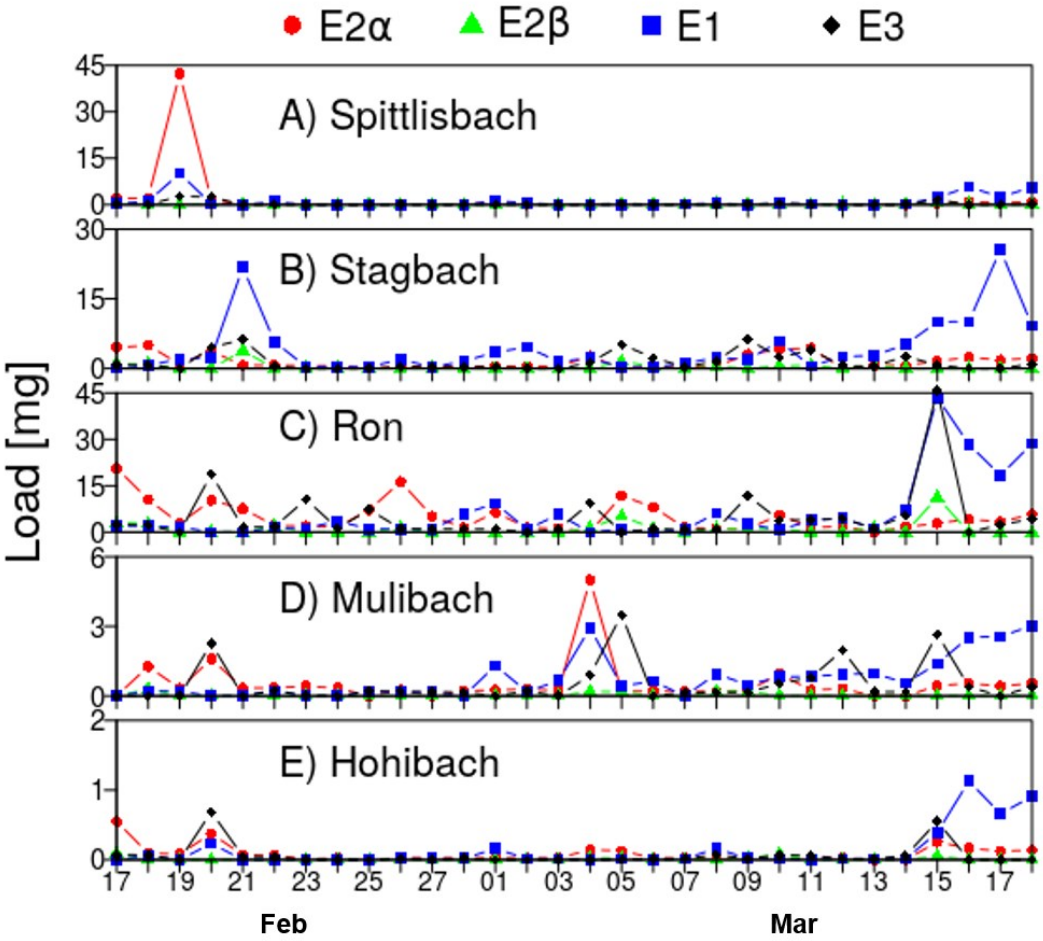

Figure S8: Daily load of 17α-estradiol (E2α, red circles), 17β-estradiol (E2β, green triangles), estrone (E1, blue squares), and estriol (E3, black diamonds) in Lake Baldegg tributaries (Spittlisbach (panel A), Stagbach (panel B), Ron (panel C), Mulibach (panel D), Hohibach (panel E)); for locations of sampling sites on the map, see Fig. 1) at the beginning of the vegetation period from February 17 to March 18, 2019. Note that the scales on the y-axes vary among the catchments.

The natural estrogen load of a day was added to the cumulative natural estrogen load of the previous measurement days to obtain the daily cumulative natural estrogen loads. Over all catchments and natural estrogens, E1 was exported in highest loads. 17α-estradiol loads exported to Lake Baldegg exceeded exported E1 loads only in the cattle dominated catchment of Spittlisbach (Table S1). Except for Spittlisbach, the entry of E2α and E3 loads to Lake Baldegg were comparable in all catchments and were minor than exported E1 loads. Exported E2β loads were the lowest in all tributaries.

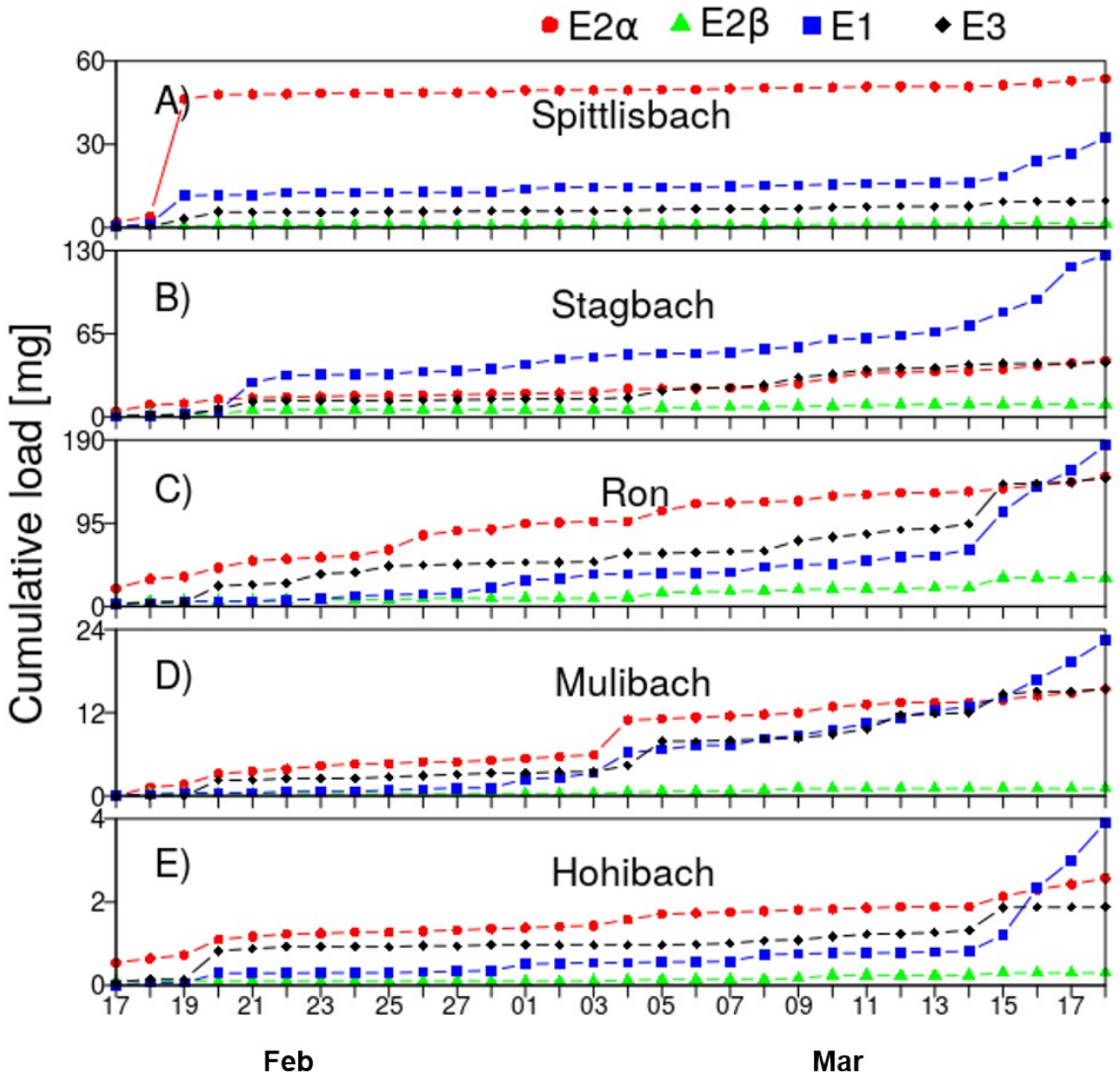

Figure S9: Daily cumulative load of 17α-estradiol (E2α, red circles), 17β-estradiol (E2β, green triangles), estrone (E1, blue squares), and estriol (E3, black diamonds) in Lake Baldegg tributaries (Spittlisbach (panel A), Stagbach (panel B), Ron (panel C), Mulibach (panel D), Hohibach (panel E)); for locations of sampling sites on the map, see Fig. 1) at the beginning of the vegetation period from February 17 to March 18, 2019. Note that the scales on the y-axes vary among the catchments.

The relative concentration in percent, meaning individual estrogen concentrations divided by the total natural estrogen concentrations (E2α+E2β+E1+E3) is shown in Fig. S10.

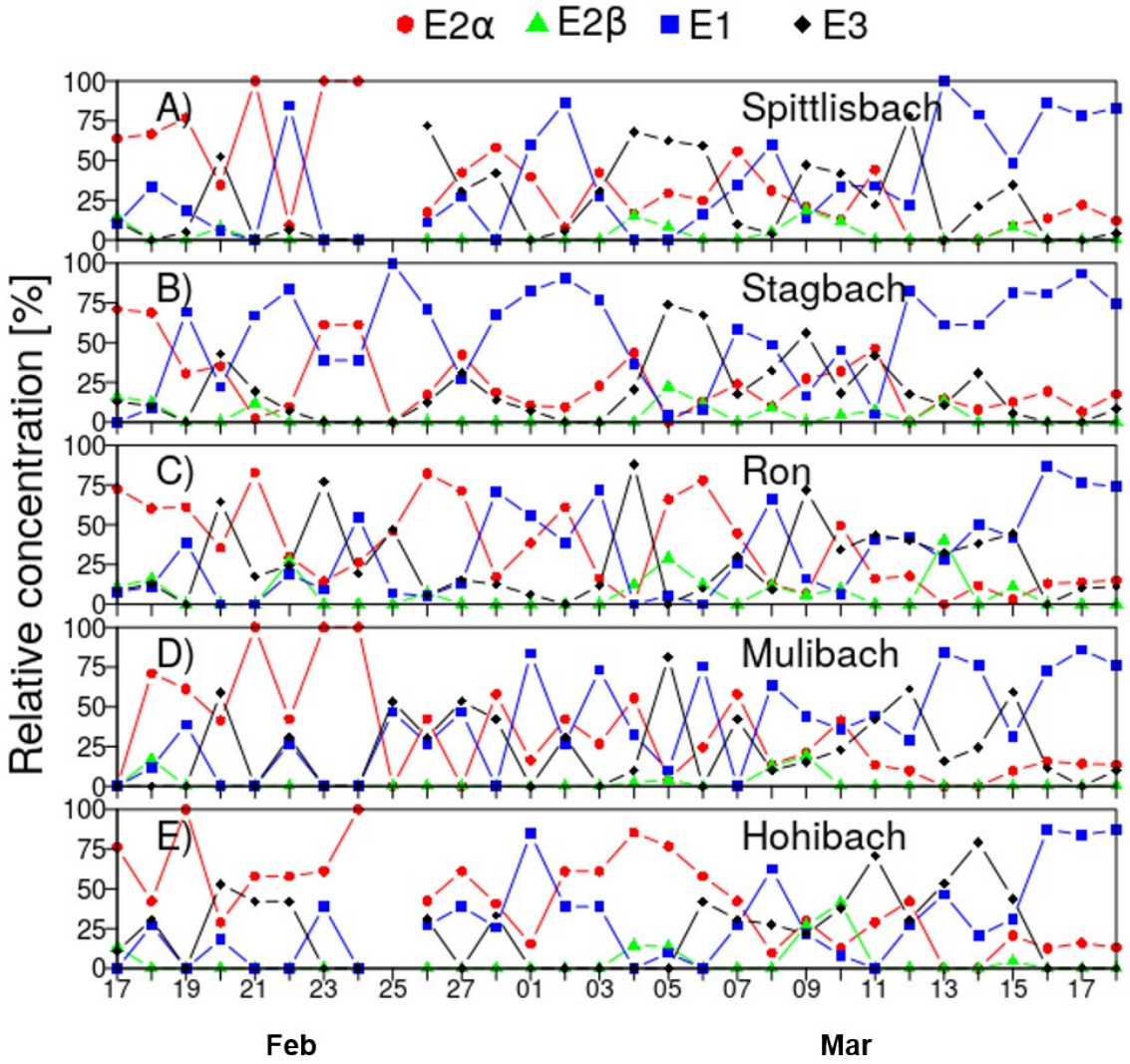

Figure S10: Relative concentrations of 17α-estradiol (E2α, red circles), 17β-estradiol (E2β, green triangles), estrone (E1, blue squares), and estriol (E3, black diamonds) to the total natural estrogen concentrations (E2α+E2β+E1+E3) in percent in Lake Baldegg tributaries (Spittlisbach (panel A), Stagbach (panel B), Ron (panel C), Mulibach (panel D), Hohibach (panel E)); for locations of sampling sites on the map, see Fig. 1) at the beginning of the vegetation period from February 17 to March 18, 2019.

S3.1.1 Statistical analyses

The results of the Shapiro-Wilk test and the skew value of natural estrogen and phosphorus concentrations and all explanatory variables are listed in Table S14. The results of the LMM are shown in Table S15.

Table S14: For natural estrogen and phosphorus concentrations in Lake Baldegg tributaries and all explanatory variables of the linear mixed-effects models p-values of the Shapiro-Wilk test and skew values. The statistical significance level was 0.05. Data was assumed to be normal distributed for -1<skew value<1.[22]

	Shapiro Wilk test (p-Value)	**Skew value**
Total natural estrogen concentration tributaries [ng/L] (E_{tot}) → log(Total natural estrogen concentration tributaries [ng/L])	$<2.2 \times 10^{-16} \rightarrow 0.02$	2.8→-0.4
17α-estradiol concentration tributaries [ng/L]→log(17α-estradiol concentration tributaries [ng/L])	$<2.2 \times 10^{-16} \rightarrow <2.2 \times 10^{-16}$	10.3→-2.3
17β-estradiol concentration tributaries [ng/L]→log(17β-estradiol concentration tributaries [ng/L])	$<2.2 \times 10^{-16} \rightarrow <2.2 \times 10^{-16}$	3.8→1.04
Estrone concentration tributaries [ng/L]→log(Estrone concentration tributaries [ng/L])	$<2.2 \times 10^{-16} \rightarrow <2.2 \times 10^{-16}$	3.2→-1.5
Estriol concentration tributaries [ng/L]→log(Estriol concentration tributaries [ng/L])	$<2.2 \times 10^{-16} \rightarrow <2.2 \times 10^{-16}$	2.6→-0.9
Total phosphorus concentration tbutaries [µg/L]→log(Total phosphorus concentration tbutaries [µg/L])	$<2.2 \times 10^{-16} \rightarrow 0.00$	5.2→0.5
Dissolved phosphorus concentration tributaries [µg/L]→log(Dissolved phosphorus concentration tributaries [µg/L])	$<2.2 \times 10^{-16} \rightarrow <3.6 \times 10^{-5}$	3.1→0.4
Daily amount of rain [mm] (rain)→log(Daily amount of rain [mm] (rain))	$1.1 \times 10^{-6} \rightarrow 1.22 \times 10^{-13}$	1.6→0.7
Daily cumulative discharge [L] (discharge)→log(Daily cumulative discharge [L] (discharge))	$<2.2 \times 10^{-16} \rightarrow 0.00$	2.6→-0.2
Livestock density of cattle (cattle)	0.04	0.9
Livestock density of pigs (pigs)	0.1	-0.8
Organically fertilized agricultural area [ha] (org. fertilized)	0.05	0.9
Surface water connected agricultural area [%] (SW-connected)	0.39	-0.1
Hydrologically contributing agricultural area [%] (hydr. contributing)	0.82	0.3

Table S15: Conditional and marginal R^2 and p-value of likelihood ratio test of LMM.

Fixed effects	Marginal R^2	Conditional R^2	Likelihood ratio test (p-value)
Log(rain) *Log(discharge)*	0.24	0.55	-
Log(rain) *Log(discharge)* *Hydr. contributing*	0.26	0.56	0.02
Log(rain) *Log(discharge)* *SW-connected*	0.27	0.56	0.03
Log(rain) *Log(discharge)* *Org. ferilized*	0.23	0.56	0.46
Log(rain) *Log(discharge)* *cattle*	0.23	0.56	0.85
Log(rain) *Log(discharge)* *pigs*	0.23	0.56	0.88

S3.1.2 Correlation between natural estrogens and phosphorus

Orthophosphate, dissolved phosphorus and total phosphorus concentrations in Lake Baldegg tributaries from February 17 to March 18, 2019 are shown in Fig. S11. Natural estrogen concentrations were correlated to dissolved and total phosphorus concentrations in tributaries (Table S16).

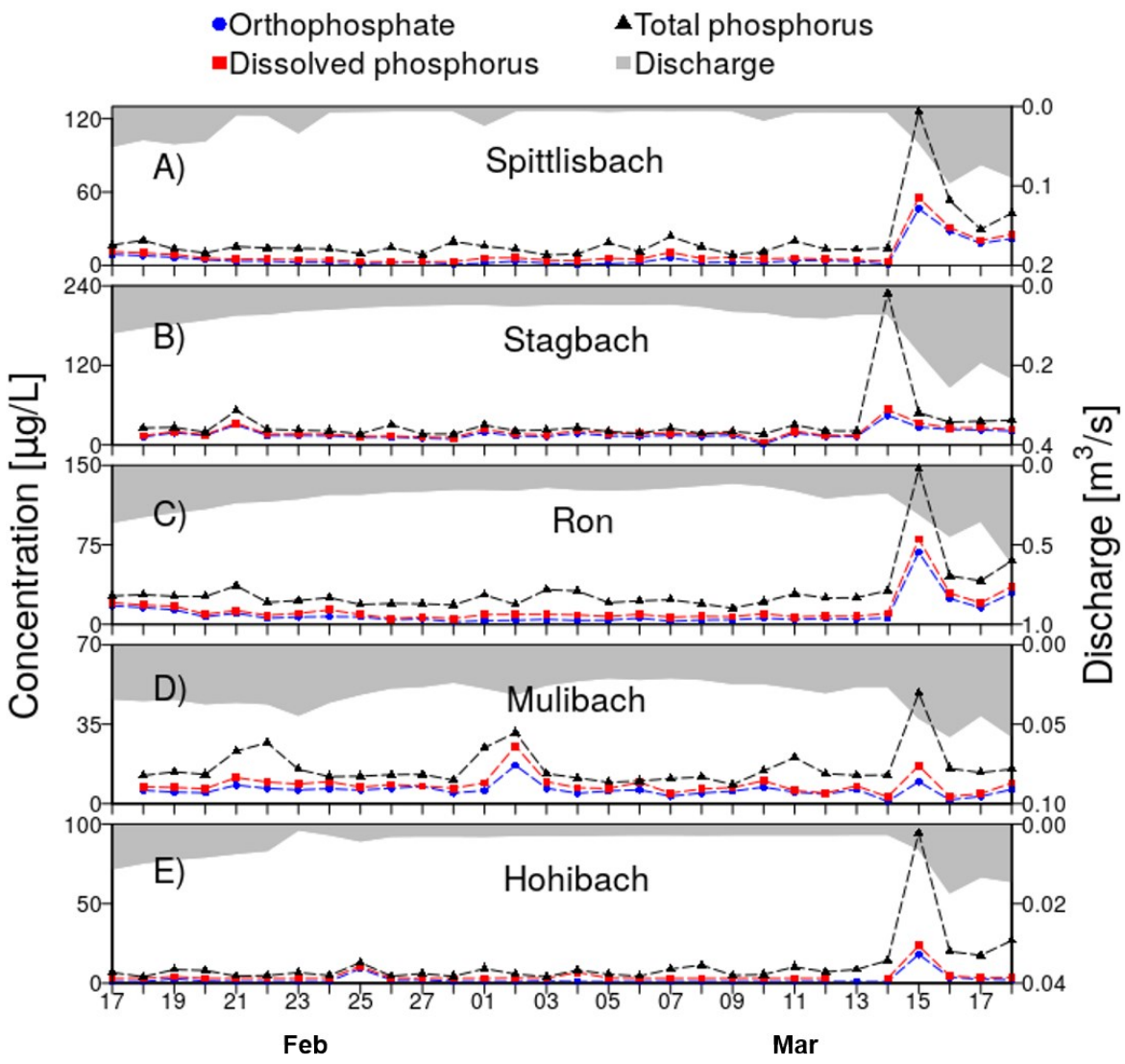

Figure S11: Concentrations of orthophosphate (blue circles), dissolved phosphorus (red squares) and total phosphorus (black triangles) in Lake Baldegg tributaries (Spittlisbach (panel A), Stagbach (panel B), Ron (panel C), Mulibach (panel D), Hohibach (panel E)); for locations of sampling sites on the map, see Fig. 1) at the beginning of the vegetation period from February 17 to March 18, 2019. Concentrations are shown on the y-axis on the left. Discharge of every tributary is indicated in grey and the corresponding y-axis is plotted on the right top down. Note that scales of y-axes differ among the tributaries.

Table S16: Pearson correlation coefficients (r) between logarithmised total natural estrogen (E2α+E2β+E1+E3), 17α-estradiol (E2α), 17β-estradiol (E2β), estrone (E1), and estriol (E3) concentrations, and logarithmised total and dissolved phosphorus concentrations in Lake Baldegg tributaries.

Pearson correlation coefficient (r)	Log(Total phosphorus)	Log(Dissolved phosphorus)
Log(Total natural estrogens (E2α+E2β+E1+E3))	0.41	0.34
Log(17α-estradiol (E2α))	0.14	0.25
Log(17β-estradiol (E2β))	-0.04	0.00
Log(estrone (E1))	0.30	0.24
Log(estriol (E3))	0.08	0.03

S3.2 Estimation of natural estrogen concentrations in tributaries including an uncertainty assessment

The sensitivity analysis showed that a change in the the natural estrogen concentration in slurry had the strongest influence on the estimated mean natural estrogen concentrations in Lake Baldegg triburatries (Table S17).

Table S17: The standard deviation relative to the mean estimated natural estrogen concentrations in Lake Baldegg tributaries as a response to a change in the mean values of the parameters of the Monte Carlo simulation by 10% ($\mu \times 0.1$) for the sensitivity analysis (non-shaded parameters and associated distributions). For parameters with a uniform distribution, the normal distribution with a standard distribution of 10% of the mean was used. Normal distribution is indicated by N(μ,σ) with mean μ and standard deviation σ. Uniform distribution between a and b is represented by Unif(a,b). Parameters which are log-normal distributed are shown with logN(μ,σ) with mean μ and standard deviation σ. Parameters and associated distributions used in the simulation are grey shaded. The parameter "Annual volume of slurry produced per cattle or pig" and the associated distribution was used in both the simulation and the sensitivity analysis.

Parameter	Relative standard deviation [%]	Associated statistical distrbution
Annual volume of slurry produced per cattle or pig	8.5	$N(\mu_{V,slurry}, \mu_{V,slurry} \times 0.1)$
Natural estrogen concentration in slurry	281.3	$logN(\mu_{c,\ slurry}, \sigma_{c,\ slurry})$
Natural estrogen concentration in slurry	47.6	$logN(\mu_{c,\ slurry}, \mu_{c,\ slurry} \times 0.1)$
Number of slurry applications per year	26.9	*Unif(2, 5)*
Number of slurry applications per year	10.3	*N(3.5, 0.35)*
Emitted fraction of natural estrogens to surface waters	51.1	$Unif(E_{F,min}, E_{F,max})$
Emitted fraction of natural estrogens to surface waters	9.4	$N(\mu_{EF}, \mu_{EF} \times 0.1)$
Cumulative discharge volume Lake Baldegg tributaries($V_{tributaries}$)	10.3	$N(V_{tributaries,}, V_{tributaries} \times 0.1)$

S3.3 Comparison of chemically and biologically determined 17β-estradiol equivalent concentrations

Only a minority of the samples, seven of 138 (=5%) collected from Lake Baldegg tributaries showed a estrogenic activity, of which five were above the previously defined quantification limit (of 0.50 ng EEQ_{bio}/L) and two samples were between LOQ and LOD (LOQ/3 of 0.2 ng EEQ_{bio}/L). The estrogenic activity of the remaining 131 samples was below LOD.

S3.4 Natural estrogens in ponds

Comparing relative concentrations in Lake Baldegg tributaries (Fig. S12A) and ponds (Fig. S12B) demonstrated different natural estrogen abundances among these surface water bodies. In tributaries, E1 was the predominant estrogen. 17α-Estradiol dominated in ponds.

Whereas in terms of median relative concentration E3 is the third natural estrogen in tributaries, it is nearly absent in ponds. 17β-estradiol relative concentrations were lowest in both tributaries and ponds.

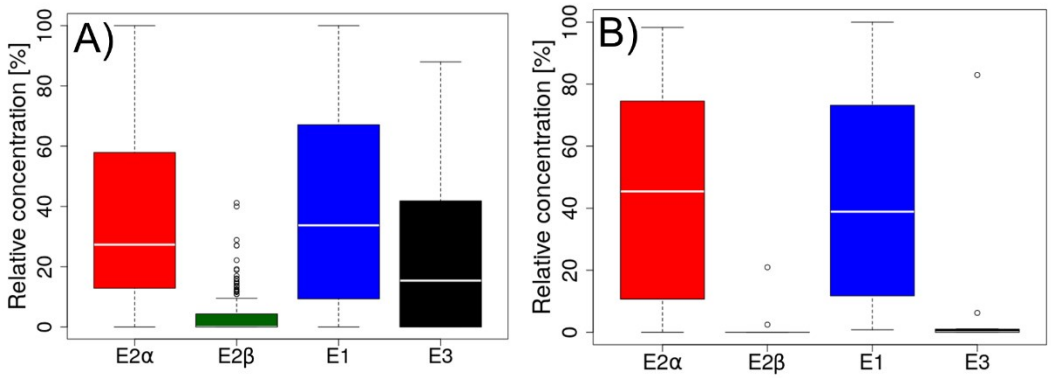

Figure S12: Boxplots of relative concentrations of 17α-estradiol (E2α, red), 17β-estradiol (E2β, green), estrone (E1, blue), and estriol (E3, black) to the total natural estrogen concentration (E2α+E2β+E1+E3) in percent in Lake Baldegg tributaries (panel A) and ponds (panel B) in the catchment of Lake Baldegg (for locations of sampling sites on the map, see Fig. 1). The white segment inside the rectangle indicates the median relative natural estrogen concentrations and whiskers above and below the box are the locations of minimal and maximal relative natural estrogen concentrations excluding any outliers. A central rectangle links the first and third quartile. Empty circles represent outliers.

In March and April 2019, we monitored 12 ponds in the catchment of Lake Baldegg. One pond was sampled weekly over a period of four weeks (Fig. S13). For every pond the chemically determined EEQ_{chem} was calculated (Fig. S14).

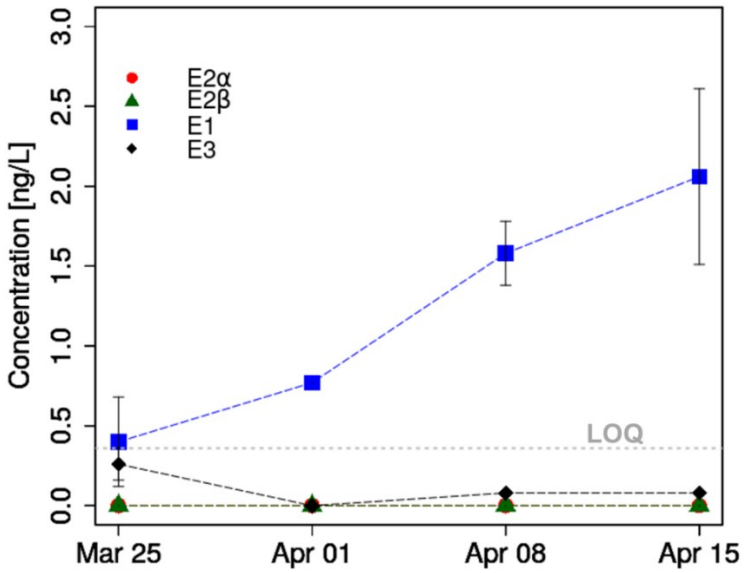

Figure S13: Concentrations of 17α-estradiol (E2α), 17β-estradiol (E2β), estrone (E1), and estriol (E3) in pond Breitholz (for location on the map, see Fig. 1) over a period of four weeks in March and April 2019. Error bars represent the standard deviations of the triplicates of samples. The grey dashed horizontal line indicates the limit of quantification (LOQ) for E3, which had the highest LOQ among all natural estrogens.

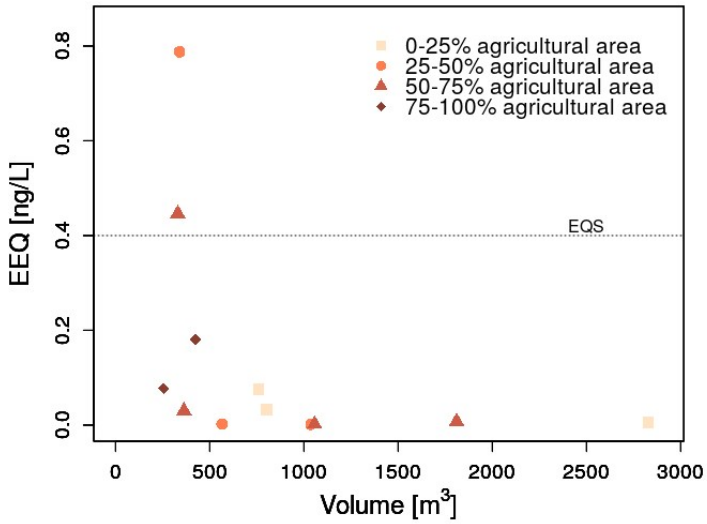

Figure S14: 17β-estradiol equivalent concentrations (EEQ_{chem}) in ponds in the catchment of Lake Baldegg plotted against the estimated volume of the ponds. The color and shape indicates the percentage of agricultural area that is in the circumference of 250 m around the ponds (light coral squares: 0-25% agricultural area, coral circles: 25-50% agricultural area, dark coral triangles: 50-75% agricultural area, brown diamonds: 75-100% agricultural area). The black dotted line represents the European Union environmental quality standard (EU EQS)[23], which is 0.4 ng/L for 17β-estradiol in surface water.

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