Ecotoxicological investigations of milking cow slurry and changes of oestrogenic compounds in the solid and liquid phase

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Abstract Due to the appearance of intensive livestock-rearing systems since the 1970s, a vast amount of liquid manure (slurry) has been produced. The application and utilization are partly regulated for this special substance, which contains urine, excrement, process water, and other chemicals, such as insecticides and disinfectants. Our research was conducted on a slurry management system of a dairy cattle farm, focusing on the investigation of the presence of oestrogenic substances and toxicity of slurry before and after using a separator technology. Yeast oestrogen screen shows in this study that the separation of the liquid and solid phases of slurry contributes to the reduction in oestrogenic substances. Based on the toxicological studies, the growth inhibition of algae was 93%, and the inhibition of the germination of higher plant seeds was 25%, without separation. After separation of the phases, growth and germination were comparable with the negative control; moreover, some stimulation was detected. Consequently, harmful substances were removed with the suspended solid phase. From the present study, we can conclude that stakeholders should support separation technology in intensive livestock-rearing systems, and ecotoxicological investigations obviously help the risk management of slurry utilization. To our best knowledge, this is the first paper presenting detailed and complex toxicological study on slurry samples.

Keywords Slurry · Dairy farm · Oestrogen · Toxicology

1 Introduction

Amongst natural elements, soil plays a significant role in food production; therefore, contamination-free, good-quality soil is highly important (Kopittke et al. 2019). The rapid growth of the agriculture industry, widespread drug utilization, and intense animal husbandry technologies are causing problems that have not been considered before (Liu et al. 2018; Oliver and Gregory 2015; Pimentel and Burgess 2013). Watering with slurry is a good solution for farmers aiming to get rid of the by-product and useful for agricultural landowners who use it as nutrient for plants. Slurry can only be released into an agricultural area in possession of a soil protection authority permit (CXXIX Act 2007) in Hungary. The authorization is issued by the Office of Agriculture Directorate and is based on soil and slurry testing. Decree 90/2008 (VII.18) of the Ministry of Agriculture and Rural Development provides that designated areas need a soil protection plan that should include detailed load calculations for the applied nutrient content. Nitrate-sensitive areas are subject to even stricter regulations, based on different decrees (Decree 27/2006 (II.7), Decree 59/2008 (IV.29)). The Nitrates Directive (Council Directive 91/676/EEC) promotes the use of good farming practices by preventing nitrates from agricultural sources and protects water quality across Europe (Goodchild 1998).

During the authorization process, the slurry is tested for its nutrient content, dissolved salts and other elements (e.g. N forms, P, K, Ca, Mg, Na, Fe, Mn, Cu, Zn), but this testing does not include ecotoxicological studies. Although the nutrient content of the slurry is high, it could contain other harmful elements (e.g. disinfectants, drug residues, hormones) that can have negative effects on plants, soil or other organisms (Adeel et al. 2017; Goeppert et al. 2015).

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According to the literature, steroid oestrogens such as estrone (E1), oestradiol (E2), estriol (E3), and synthetic oestrogen (EE2) can be found everywhere in the soil as a result of watering with slurry and manure applied to agricultural fields originating from intense animal husbandry operations (Goeppert et al. 2014; Zheng et al. 2008; Adeel et al. 2017). The emissions of livestock kept in the USA and the European Union together exceed 83 000 kg of natural oestrogens (E1, E2 and E3) annually, which is double the total of human emissions (Adel et al. 2017). Oestrogens (E2, E1, and E3) are predominantly female hormones responsible for the healthy maintenance of reproductive tissues, like breast, skin, and brain. In men, the testicles produce small amounts of oestrogen (Lai et al. 2000). The oestrogenic potential of different chemicals is most often expressed as the relative potential to the 17 β -E2 reference compound. The quality of the compounds plays a determinative role, as each oestrogenic compound can have a different effect on the receptors. From Coldham et al., it is well known, that compared to the oestrogenic potential of 17 β -E2 (100%), the receptor affinity of 17 α -EE2 is 88.8%, E1 is 9.6%, 17 α -E2 is 5.25%, E3 is 0.63%, and BPA is 0.005% (Coldham et al. 1997). Oestrogenic substances have several adverse effects for animals and human, such as distracting the hormonal system, reducing the efficiency of the immune system, causing developmental disorders, and the most common negative effect is dysfunction of the reproductive system, which can lead to reduced fertility and changes in sexual behaviour (Kumar et al. 2020). Sex drive and responsiveness, gender identification, and sexual preference are all influenced by hormones and therefore by endocrine-disrupting chemicals. (Mhaouty-Kodja et al. 2018; Lopez-Rodriguez et al. 2021).

Our research was conducted at a dairy cattle farm using a slurry management system, focusing on the investigation of the presence of oestrogenic chemicals in the slurry due to intense husbandry. We monitored the changes in the quantity of the oestrogenic substances, the storage effects and treatment procedures over a wide range of time periods for twelve months starting in January 2017. Additionally, we conducted ecotoxicological studies on the same samples using different test organisms (soil bacteria, *Daphnia magna*, different plant seeds, algae) providing a detailed and complex toxicological study on slurry samples.

2 Materials and methods

2.1 The origin of the investigated samples

Samples originated from a dairy cattle farm in Komárom-Esztergom County, Hungary. The average number of animals at the farm is 1200, including 600 dairy cattle and 600

dry cows, heifers, and calves. The manure from the barn's individual stalls is regularly rinsed with water and transferred to a storage pool. At specific time intervals, if the pre-pool storage is full, the liquid and the suspended solid parts of this material are separated using a machine called a separator. Separation can be performed by settling tanks or by forced settling with the use of centrifuges. This latter technique allows to reduce the settling time for separation using centrifugal force. The applied device is a vertical decanter centrifuge which in operation separates solids and liquids at the wall with rotations at high speed. The solid particles then conveyed towards the conical end of the structure while the supernatant enters to bowl-shape cylinder (Hjorth et al. 2010). The solid part is treated as bedding manure after further processing, while the liquid part is stored in the pool, stirred continuously with two high-performance stirrers and finally delivered to the cropland. The irrigation period runs from early spring until late October.

In 2017, we sampled two pools in each season. Four sample were taken from the fresh slurry storage pool and four sample from the pool, which is the endpoint of the separation process, where the slurry usually stored for 30 days. Samples were taken each time from the four edges of the pool (1 L each) and were combined. In both pools, the slurry was mechanically mixed, therefore samples deemed as homogenous and representative samples. In the spring season, the separator was out of order; consequently, the 30-day material was not separated.

Samples were collected in disposable, sterile polypropylene Falcon tubes, which were free of DNase, RNase, endotoxin and metal, were freezable down to -80 °C and were resistant to chemicals. Samples were placed in the refrigerator and processed within 4–6 days after sampling.

2.2 Yeast oestrogen screen for the investigation of oestrogenic effect

The samples were centrifuged in 50-mL Falcon tubes for 20 min at a speed of 4200 rotations per minute (rpm) at a constant temperature of 4 °C. Endocrine-disrupting chemicals were extracted from the 30 mL supernatant using solid-phase micro extraction (SPE). First, the extraction cartridge (Strata $\times$ 200 mg/6 mL from Phenomenex, USA) was conditioned using a mixture of 8 mL methanol and 8 mL water with a 95:5 ratio, completing a regular rinse with technical grade methanol solution. In the second step, 30 mL of liquid slurry supernatant was passed through the cartridge. In the third step, rinsing was performed with 10 mL methanol–water (1:1) and 10 mL acetone–water (2:1). After a few minutes of drying, the retained components were eluted in 5 mL methanol. After elution, 200 μ L

of sample was dissolved in 1800 µl methanol, which was a tenfold dilution. Then, further $50 \times$, $100 \times$, $500 \times$, $1000 \times$, $5000 \times$, and $10\,000 \times$ dilutions were prepared for yeast testing. Two (2) grams of the sediment (from centrifugation) was mixed with 10 mL methanol and sonicated for 30 min at 30°C and then centrifuged for 10 min at 4 °C at 2 000 rpm velocity. The supernatant was further diluted, as in the liquid phase ($10 \times$ – $10\,000 \times$), and then used for testing.

As discussed above, our environment is polluted from several sources by substances, that can bind to human oestrogen receptor. The chemical diversity of these substances is high, which makes their analysis very difficult as different analytical methods are required for their detection. Even very detailed analysis of a particular sample, it cannot be completely ruled out that any of the oestrogenic substances remain undetected. This problem is bridged by the methods which detect the oestrogenic substances from the effect side such as the in vitro reporter gene assay, the yeast oestrogen screen (YES). Additionally, it has a number of advantages over other similar detection systems, such as the absence of endogenous steroid hormone receptors and consequent lack of complex interactions between the oestrogen receptor (ER) and other receptors (Routledge and Sumpter 1996; Purvis et al. 1991).

Because of the reasons above, oestrogenic activity of the sample extracts was evaluated using the recombinant yeast strain *Saccharomyces cerevisiae* BJ3505, according to the protocol ISO 19040–1:2018. The yeast continuously produces the human oestrogen receptor. When the human oestrogen receptor interacts with oestrogen or with oestrogen analogue molecules in the cytoplasm, it is activated. The activated receptor activates the lacZ operon on the plasmid, starting the production of the β -galactosidase enzyme in proportion to the amount of the oestrogen or oestrogen analogue molecules present in the sample. The activity of the produced β -galactosidase enzyme was measured using ultraviolet–visible spectroscopy (LabSystems Multiskan MS) at 580 nm, where β -galactosidase catalyses the hydrolysis of the galactoside analogue chlorophenol red- β -D-galactopyranoside (CPRG) and converts the yellow-orange CPRG substrate into the red chromophore chlorophenol red, yielding a dark red solution (Routledge and Sumpter 1996; Purvis et al. 1991).

For the analysis of the oestrogenic activity, 10-µL aliquots of the sample extract were transferred to the wells of a sterilized 96-well optical flat-bottom microtitre plate (Nunc, Germany), and the solvent was allowed to evaporate until dry. The wells were then supplied with 120 µL of the assay medium containing yeast cells, and the covered plates were incubated at 30 °C in an incubator (PLO-EKO Aparatura) for 1 day. Then, 30 µL of each well of the test plate was transferred to a new 96-well plate, and 50 µL

reaction mixture containing CPRG and Lyticase was added; the plates were then incubated for one more hour. The colour development was measured at 580 nm, and the turbidity of the yeast cell biomass was read at 620 nm (LabSystems Multiskan MS).

In parallel, each plate contained the concentrations of the standard E2 (0.66 ng L^{-1} to 500 ng L^{-1}) as a positive control and negative control wells consisting of either methanol alone or processed distilled water, as well as blank wells containing no organism but treated in the same way as the other replicates in the sample. Each test substance was analysed four times.

Based on the results (initial and final yeast cell density and colour change at 580 nm), the relative growth (620 nm) to assess the potential toxic effects of the sample and the average corrected absorbance (580 nm) were calculated using Microsoft Excel. Subsequent statistical evaluation and concentration–response curves were constructed using a Web-based tool (www.elisaanalysis.com). The standard curve calibration was performed using a 4- or 5-parameter logistic regression model (Findlay and Dillard 2007). For the determination of E2-oestradiol equivalents (EEQ), the corrected absorbance of the sample extracts was interpolated in the linear range of the corresponding oestradiol standard curve (Hong 2012).

2.3 Toxicological tests

Ecotoxicological tests characterize potential adverse effects that a slurry causes to a terrestrial or an aquatic receptor. For the toxicological evaluation of the slurry 4 test organisms were selected paying attention to their different place in the phylogenetic tree. As a result, 2 terrestrial (bacteria and plant seeds) and 2 aquatic (algae and crustacean) receptor were chosen. Relative chemical hazards to terrestrial organisms do not necessarily follow the same patterns as that seen with aquatic organisms, necessitating separate testing and assessment schemes. Monetary and ethical considerations make difficult to conduct toxicity tests on vertebrates (“National Research Council” 2014; Parry 1998).

Each toxicological test was performed on undiluted samples and on $10 \times$, $50 \times$, $100 \times$ and $500 \times$ dilutions. The determination of these dilution factors was calculated from earlier oestrogen content studies. Samples for the toxicology tests were collected only in the summer period, not in all seasons, since the liquid slurry is mainly applied in the summer period during row-crop cultivation and irrigation purposes.

The oestrogen content (EEQ) of the investigated samples were $390 \pm 40 \text{ ng L}^{-1}$ before separation and $3 \pm 0.3 \text{ ng L}^{-1}$ after separation determined by yeast screen. The slurry tested by us can be said to be average in

terms of nutrient content. Before separation the characteristics of the slurry were as follows: dry matter content: 20 g L^{-1} ; pH: 7.15, total nitrogen content: 0.98 g L^{-1} ; ammonium-nitrogen: 0.25 g L^{-1} ; nitrite-nitrate-nitrogen: $< 0.50 \text{ g L}^{-1}$; phosphorus: 0.23 g L^{-1} ; potassium: 0.63 g L^{-1} . After separation the contents were modified as follows: dry matter content: 0.9 g L^{-1} ; pH: 7.14; total nitrogen content: 0.39 g L^{-1} ; ammonium-nitrogen: 0.07 g L^{-1} ; nitrite-nitrate-nitrogen: $< 0.30 \text{ g L}^{-1}$; phosphorus: 0.13 g L^{-1} ; potassium: 0.41 g L^{-1} . As it is visible from the data the separator reduced the dry matter content by 95.5%, total nitrogen content by 60.2%, ammonium-nitrogen by 72.0%, nitrite-nitrate nitrogen by 40.0%, phosphorus by 43.5% and potassium by 35.0% and pH did not change. Anti-inflammatory drugs such as marbofloxacin (1700 mg per month), ketoprofen (2300 mg per month), prednisolone (200 mg per month), antibiotics such as udder infusions, ampicillin (2500 mg per month), cloxacillin (200 mg per month), cefquinom (100 mg per month), novobiocin (150 mg per month), benzylpenicillin procaine (100 mg per month), dihydrostreptomycin (100 mg per month), neomycin (150 mg per month), cefotiofur (6000 mg per month), oxytetracycline (12,000 mg per month) and sex-inducing and synchronizing products such as cloprostenol (22 mg per month), prostaglandin F2 alpha (7100 mg per month), D-Phe6-Gonadorelin (180 mg per month) were used regularly at the investigated farm. Furthermore, parlour acid- (30–32% hydrochloric acid, 200 L per month, 20% phosphoric acid, 100 L per month) and/or alkaline-based (15% sodium hypochlorite, 500 L per month) chemicals also applied for the disinfection of milking cows and trotter care products. These chemicals also appear in slurry samples.

2.3.1 *Azotobacter agile* and *Pseudomonas fluorescence* soil toxicity test

Azotobacter agile and *Pseudomonas fluorescence* test organisms are contamination-sensitive soil bacteria (Gruijs et al. 2001). These bacteria have the ability to fix molecular nitrogen and therefore increase soil fertility and stimulate plant growth. *Azotobacter* species are widely used in agriculture, particularly in nitrogen biofertilizers (Neeru 2000). *Pseudomonas fluorescence* are commensal rhizosphere bacteria that help plants attain key nutrients, degrade pollutants, and suppress pathogens via antibiotic production. Plants provide nutrients for these organisms and shelter them against stressful environments (Paulsen et al. 2005).

The aim of these tests was to detect any growth inhibition of these soil bacteria. The agrochemical properties of our reference test soil were as follows: humus content $1.25 \text{ m m}^{-1}\%$, binding (KA) 31, which corresponds to

sandy loam soil, $\text{pH}_{(\text{KCl})}$ 7.2 neutral pH, carbonated lime content $4.2 \text{ m m}^{-1}\%$, weakly calcareous, nitrite-nitrate nitrogen 25 mg kg^{-1} , phosphorus 110 mg kg^{-1} , and potassium 140 mg kg^{-1} . First, the test soil was air-dried on a flat surface, and the water holding capacity was determined as follows: 90 g soil sample was mixed with 50 mL distilled water in a graduated cylinder and was allowed to stand for 24 h until the soil–water mixture reached equilibrium. A microsieve cylinder and a micropipette were used to remove the water from the surface of the soil and transfer the retrieved water (S) into a graduated cylinder. The volume of water (V_{sat}) needed for complete hydration of the test soil (90 cm^3) was calculated using the following formula: $V_{\text{sat}} = 50 - S$. During the test, 210 cm^3 test soil was mixed with 65 mL slurry ($V_{\text{sat}} = 28 \text{ ml}$). Soil extraction was performed using high-purity water (100 mL water was added to 10 g soil), and dilution series were prepared ($2 \times$, $5 \times$, $10 \times$, $25 \times$, $50 \times$, $100 \times$).

Tests were performed in microtitre plates in three repetitions, in which each well contained 0.2 cm^3 modified Fjodor Durov broth containing triphenyltetrazolium chloride (TTC), bacterial culture and 0.05 cm^3 sample. Positive (10 mg/L Cu solution) and negative (high-purity water, ‘Herco’) controls were also included. Samples were incubated for 48 h at 28°C (Pol_Eko_Aparatura 4.81 Incubator). The bacterial activity is visible when the colourless TTC changes into red triphenyltetrazolium formazan (TTF). Red indicates no growth inhibition (+), pale red indicates medium growth inhibition ($\pm$) and colourless broth indicates total growth inhibition (–) (MSZ 21,978:30).

2.3.2 *Daphnia acute immobilization test*

Daphnia magna Straus juveniles younger than 24 h old are sensitive to environmental changes, including chemicals. Newborn crustacea were separated from the culture stock of the National Public Health Center, Budapest and were used for the investigations. Tests were performed in 2 replicates, and 10–10 juveniles were placed in 50 mL test medium and incubated for 48 h at 22°C in an air-conditioned room. The immobilization was read every 24 h. Quality controlled tap (drinking) water, aerated for 7 days, was used as a negative control and for dilution. Dissolved oxygen (DO) and pH were measured at the beginning and at the end of the test in order to control the testing circumstances (pH: 7.2–9.4 and $\text{DO} > 2 \text{ mg L}^{-1}$). The *Daphnia acute immobilization test* is valid if the number of immobile animals is less than 10% in the negative control and the sensitivity of the *Daphnia magna* culture against potassium dichromate reference solution is in the range of $0.6\text{--}2.1 \text{ mg L}^{-1} \text{ EC}_{50\text{i}}$ (24 h) (ISO 6341).

2.3.3 Algae growth inhibition test

The aim of this test was to detect cell growth inhibition of the green algae *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák (origin: Culture Collection of Algae and Protozoa, UK; CCAP 278/4) in different dilutions of the slurry. Fifty millilitre samples were tested in Erlenmeyer flasks, and each dilution was analysed in three replicates. Samples were spiked using 800 µL *Pseudokirchneriella subcapitata* cell culture in the phase of exponential growth (10^5 cell mL⁻¹). The starting algae cell density was determined using a Burkner counting chamber (Hirschmann EM Techcolor, Tiefe Depth Profondeur 0.0025 mm²; 0.100 mm) and an optical microscope (Leica Microsystems, Leica DM 2500). The algae cell optical density at 750 nm was determined using a spectrophotometer (Metertech Inc., SP-830 Plus) and the starting optical density was 0.02, which corresponds to a 0-h OD. Negative control (Zhender-8 broth) and blank flasks that contained no organism but were treated in the same way as the other replicates in the sample were also included. Samples were incubated for 72 h with continuous shaking (speed of 100 rpm) and lighting (8,500 lx) at 24 °C (Witeg Labourtechnik GmbH, Witeg WIS-RL Shaking Incubator). On the last day, cell density-based growth inhibition (optical density at 750 nm) was measured using a spectrophotometer (Metertech Inc., SP-830 Plus) calibrated to the blank solutions. This toxicity test is regarded as valid if the average daily algae cell growth is at least $1.4 \times$ in the negative control, the value of pH does not change more than 1.5 units, the coefficient of variation of the negative control's growth rate is not more than 5% and the sensitivity of the *Pseudokirchneriella subcapitata* culture against potassium dichromate reference solution is within the range of 1.19 ± 0.27 mg L⁻¹ (ISO 8692).

2.3.4 Phytotoxkit microbiotest

A Phytotoxkit microbiotest (MicroBioTests Inc., TK62 L Phytotoxkit) was used to test agricultural seed germination and the growth of young roots. Both monocotyledonous (*Sorghum saccharatum*; *Triticosecale*) and dicotyledonous (*Sinapis alba*; *Lepidium sativum*; *Fagopyrum esculentum*) plants were selected for testing. First, the test soil was air-dried on a flat surface, and the water holding capacity was determined as described above. During the test, 90 cm³ test soil was mixed with 30 mL slurry or with its dilutions. Ten seeds per plate were positioned at equal distance near the middle ridge of the test plate; the plates were then covered and placed vertically in a holder box and incubated at 25 °C for 3 days (Pol_Eko_Aparatura 4.81 Incubator). After the incubation time, the lengths of the roots were measured, and the germination rate and growth inhibition

were calculated as percentages compared to the control sample. For control plates, distilled water was used. The seedling growth test is regarded as valid if the germination average is at least 70%, and the average length of the roots in the control is at least 30 mm (MicroBioTest 2018).

2.3.5 Data analysis

A relationship between the observed effects on immobility and growth rates to the exposure concentrations were established and the US Environmental Protection Agency Probit Program Ver 1.5 was used to calculate the ED (effective dilution) values and their 95% confidence intervals for this study (EPA 1999). Median effect dilution (ED₅₀) and effective dilution at 10% inhibition (ED₁₀) were calculated for *Daphnia* immobilization test, bacteria and algae test, respectively. The ED₅₀ refers to the dilution of a substance that induces a response halfway between the baseline and maximum and ED₁₀ refers to the dilution that causes the measured effect in 10% of organisms after the exposure time. In case of phytotoxkit microbiotest values representing a no-effect level (NOEL) were also given. NOEL is the highest dose or exposure level of the slurry that produces no noticeable (observable) toxic effect on tested plants (ISO 8692, ISO 6341, MicroBioTest 2018).

3 Results

3.1 YES test

During the studies, the determination of the content of the oestrogenic substances in our samples was performed in 7 different dilutions ($10 \times$, $50 \times$, $100 \times$, $500 \times$, $1,000 \times$, $5,000 \times$, $10,000 \times$). The highest dilution at which all (16) samples gave negative results was $10,000 \times$. Testing of the samples started at a concentration close to the original concentration, tenfold ($10 \times$). When the $10 \times$ dilution gave a negative result, the concentrated samples were tested (Table 1). In pharmacology, an agonist is a substance that mimics the action of a hormone by binding to the same receptor, causing a biological response, while an antagonist does not cause a response. As the processed samples may contain a very wide range of chemical substances at various concentrations, increasing the dilution may dilute certain antagonist compounds that bind to the desired human oestrogen receptor (Lehel and Laczay 2011). We assume that this is the explanation for the phenomenon that different dilutions of samples gave different results. Therefore, the average values were used for further calculations considering the deviations, respectively, (Table 1).

Table 1 Results of the yeast oestrogen screen

Code	Sample variety	Storage days	Seasons	EEQ 5000 × ng/L 95% conf	EEQ 1000 × ng/L 95% conf	EEQ 500 × ng/L 95% conf	EEQ 100 × ng/L 95% conf	EEQ 50 × ng/L 95% conf	EEQ 10 × ng/L 95% conf	EEQ CC ng/ L 95% conf	Average ng/L 95% conf	LID
K1	liquid	1 day	Winter	< LOD	< LOD	1731 ± 121	730 ± 52	387 ± 35	206 ± 19	NA	763 ± 57	1000 ×
K1x	sludge			< LOD	< LOD	1538–1924	647–813	331–443	176–236		733–793	
K3	liquid		Spring	15,502 ± 170	3245 ± 227	< LOD	501 ± 34	413 ± 25	332 ± 32	NA	416 ± 30	500 ×
K3x	sludge			< LOD	< LOD	12,049–18,955	447–555	373–453	281–383		397–435	
K5	liquid		Summer	< LOD	< LOD	1207 ± 72	643 ± 46	594 ± 60	469 ± 42	NA	3610 ± 2 623	10,000 ×
K5	sludge			< LOD	< LOD	1092–1322	570–716	499–689	402–536		2502–4718	
K7	liquid		Autumn	< LOD	< LOD	1416 ± 99	934 ± 93	948 ± 92	1144 ± 102	NA	1111 ± 97	1000 ×
K7x	sludge			< LOD	< LOD	1258–1574	786–1082	802–1094	1029–1259		1059–1163	
K8	liquid	30 day	Winter	< LOD	< LOD	< LOD	441 ± 39	340 ± 50	305 ± 20	NA	362 ± 36	500 ×
K8	sludge			< LOD	< LOD	379–503	260–420	238 ± 19	273–337	NA	339–385	
K7	liquid			< LOD	< LOD	< LOD	< LOD	208–268	294 ± 26	NA	266 ± 23	100 ×
K7x	sludge			< LOD	< LOD	< LOD	< LOD	558 ± 45	253–335		247–285	
K8	liquid			< LOD	< LOD	< LOD	365 ± 49	203 ± 17	176–230	NA	376 ± 37	500 ×
K8x	sludge			< LOD	< LOD	< LOD	287–443	486–630	176–230		352–400	
K2	liquid		Spring	< LOD	< LOD	< LOD	< LOD	680 ± 56	647 ± 58	NA	664 ± 57	100 ×
K2x	sludge			< LOD	< LOD	< LOD	< LOD	591–769	555–739		616–712	
K4	liquid		Summer	< LOD	< LOD	< LOD	< LOD	32 ± 5	59 ± 5	19 ± 1	37 ± 5	50 ×
K4x	sludge			< LOD	< LOD	< LOD	< LOD	24–40	51–67	17–21	34–40	
K6	liquid		Autumn	< LOD	< LOD	< LOD	< LOD	868 ± 73	268 ± 24	NA	568 ± 49	100 ×
K6x	sludge			< LOD	< LOD	< LOD	< LOD	752–984	230–306		527–609	
				< LOD	< LOD	1149 ± 137	990 ± 97	555 ± 42	329 ± 31	NA	756 ± 77	1000 ×
				< LOD	< LOD	931–1367	836–1144	488–622	280–378		715–797	
				< LOD	< LOD	2809 ± 27	753 ± 74	682 ± 54	413 ± 32	NA	1165 ± 47	1000 ×
				< LOD	< LOD	2766–2852	635–871	596–768	362–464		1140–1190	
				< LOD	< LOD	< LOD	< LOD	< LOD	1 ± 0.2	5 ± 0.4	3 ± 0.3	50 ×
				< LOD	< LOD	< LOD	< LOD	< LOD	-2.18 – 4.18	-1.36 – 11.36	0.49 – 5.51	
				< LOD	< LOD	< LOD	< LOD	< LOD	70 ± 6	21 ± 1	46 ± 4	50 ×
				< LOD	< LOD	< LOD	< LOD	< LOD	60–80	19–23	43–49	
				< LOD	< LOD	< LOD	< LOD	< LOD	1 ± 0.2	3 ± 0.3	2 ± 0.3	50 ×
				< LOD	< LOD	< LOD	< LOD	< LOD	-2.18 – 4.18	-1.77 – 7.77	-0.51 – 4.51	
				< LOD	< LOD	< LOD	< LOD	< LOD	29 ± 2	54 ± 4	42 ± 2	50 ×
				< LOD	< LOD	< LOD	< LOD	< LOD	26–32	48–60	40–44	

NA not available, LOD limit of detection, LID lowest ineffective dilutions, EEQ estradiol equivalent, 95% conf.: ED values with their respective 95% confidence limits

3.1.1 One-day samples without separator

The autumn and summer supernatant fraction of the centrifuged liquid slurry contained oestrogen-derivative (ED) materials at the lowest concentrations (558 ± 45 and 441 ± 39 ng L⁻¹). Similarly, in the sediment phase, the summer samples showed the lowest oestrogenic compound levels (294 ± 26 ng L⁻¹). The values of the spring samples (separator was out of order) were extremely high compared with the samples from other seasons (liquid 15.502 ± 2.170 ng L⁻¹, sediment 1.416 ± 99 ng L⁻¹) (Fig. 1).

3.1.2 30 days after separator

Similarly, for the one-day samples, the autumn and summer fractions of the centrifuged liquid slurry showed the lowest ED concentration (1 ± 0.2 – 5 ± 0.4 ng L⁻¹), while higher concentrations were observed in the spring samples (1.149 ± 137 ng L⁻¹). In the sediment, the oestrogenic compound level in the autumn samples was the lowest (54 ± 4 ng L⁻¹), while in the spring samples (separator was out of order) were the highest (2.809 ± 27 ng L⁻¹). The spring values are also extremely high in this case (Fig. 2).

3.2 Toxicological tests

3.2.1 One-day samples without separator

The two soil microbiology tests and the *Daphnia* acute immobilization test were considered valid. The percentage *Daphnia* immobilization of the controls was less than 10% (0%), and the 24-h EC₅₀ of the potassium dichromate was within the range of 0.6 mg L⁻¹ to 2.1 mg L⁻¹

(= 1.56 mg L⁻¹). In the soil toxicity tests, the positive control (10 mg L⁻¹ copper solution) showed total inhibition. These three tests did not show any negative effect of the slurry on the test organisms.

The algae growth inhibition tests were also considered valid. The biomass in the control after the 72-h test period was $27 \times$, and the pH in the different dilutions did not change by more than 1.5 during the test period. Preliminary testing using the algae growth inhibition test showed that the dilutions used ($10 \times$, $50 \times$, $100 \times$ and $500 \times$) were not suitable for further calculations, because the differences between the undiluted, original sample and the next dilution ($10 \times$) were exceptionally high. The growth inhibition in the undiluted liquid slurry was 93%, while in the $10 \times$ dilution, it was only 5% (considered as no real inhibition). For the reasons above, $2 \times$, $4 \times$, $6 \times$, and $8 \times$ dilutions were further tested. In the undiluted slurry, there was 93% inhibition, the $4 \times$ dilution showed 67% inhibition and the $10 \times$ dilution gave similar results as those of the control sample. Calculated ED₁₀ is at $9.3 \times$ dilution, 95% lower confidence limit 37.7, 95% upper confidence limit 6.1. Calculated ED₅₀ is at $4.1 \times$ dilution, 95% lower confidence limit 6.3, 95% upper confidence limit 2.2.

Based on the Phytotoxkit microbiotest, all of the seeds in the control reached the limit root length (30 mm), and the germination rate was more than 70%; thus, the tests were considered valid. Using undiluted slurry, 14–45% inhibition was measured for *Sinapis*, *Lepidium*, *Fagopyrum* and *Triticosecale*. The $10 \times$ dilutions gave almost the same results as the undiluted sample, but the $50 \times$ dilutions in *Sinapis*, *Lepidium*, and *Fagopyrum* and the $100 \times$ dilutions in *Triticosecale* gave negative results. The *Sorghum* seed was not sensitive, and the undiluted slurry

Fig. 1 Seasonal changes of EEQ-s of 1-day slurry samples in the liquid and suspended solid phase

Fig. 2 Seasonal changes of EEQ-s of 30-day slurry samples in the liquid and suspended solid phase

stimulated the growth of this plant (30% stimulation) (Table 2).

3.2.2 30-day samples after separator

The *Daphnia* acute immobilization test and the two soil microbiology tests were considered valid, as described above, and did not show any negative effects of slurry on the test organisms.

The algae growth inhibition test using undiluted or diluted slurry gave negative results. The relative algae cell growth was 97.5% in undiluted slurry, and we consider this to be under 10% inhibition (ED_{10}), which is the non-observed effect level (NOEL). We measured 2.5% inhibition and 3–16% stimulation in the dilution series. The 16% stimulation had a positive effect on algae growth.

In the case of the Phytotoxkit microbiotest, all of the seed tests were considered valid, as described above. *Sorghum* showed stimulation in each dilution, and the maximum stimulation was 60% in the undiluted sample. *Lepidium* and *Sinapis* showed 53% and 18% inhibition at a $10 \times$ dilution, respectively, but all other dilutions had no positive or negative effect on germination. The growth of other plants (*Fagopyrum*, *Triticosecale*) was near the control in each dilution (Table 2).

4 Discussion

4.1 Oestrogenic substance content

In the case of the 30-day samples, it can clearly be seen that 71–95% of the overall oestrogenic substance is absorbed to the suspended solid phase. During storage, the solid phase

absorbed the ED components, and the absorbed amount exceeded that of the liquid phase. Sorption experiments with pig slurry separation technique state that the separation of the liquid slurry enhance the infiltration of the oestrogen compounds to the soil matrix, therefore the sedimentation process involve E2 reacting with the carbon content of the crude fibre fraction of the slurry. By physical separation (sedimentation and centrifugation process) of the liquid matter, 50–75% of E2 can be absorbed in solid particles (Mostofa et al. 2012). The oestrogens are retained onto sludge due to their high adsorption (Louros et al. 2019). A separator, therefore, can highly decrease the oestrogenic effect of the liquid slurry, not only making the emission of the slurry easier but also delivering a safer substance into the environment. This conclusion is also supported by the results of the 30-day spring samples when the separator was out of order, and the oestrogenic concentration of untreated samples showed an extremely high level. To clean sewage or manure from oestrogens physical treatments such as coagulation, activated carbon and membrane separation or chemical treatments identical to advanced oxidation processes (AOPs), like ozone, ultraviolet degradation, manganese oxide, ferrate, chlorination, titanium dioxide, fenton, sonolysis, can be used. Although these treatment techniques will surely reduce the oestrogens, they represent a large financial cost in addition to environmental impact caused by increasing energy consumption and CO_2 emission (Nazari and Suja 2016).

In nitrite-sensitive areas, the liquid slurry emission limit is approximately 180 m^3 per ha (considering the 170 kg per ha approved limit for nitrogen), as determined by Decree 27/2006 (II.7). If we calculate the average amounts of oestrogen content of the liquid and the solid phases of the slurry (for the 1-day samples; liquid: $4\,560 \text{ ng L}^{-1}$ and

Table 2 Relative root growth (mm and %) in non-separated and separated samples

Tests organism	Relative root growth in non-separated samples					Relative root growth in separated samples				
	Control	Undiluted	10 ×	50 ×	100 ×	Control	Undiluted	10 ×	50 ×	100 ×
<i>Sinapis alba</i>	44.9 ± 19 mm	36.0 ± 26 mm/ 80%	35.4 ± 24 mm/ 79%	45.4 ± 24 mm/ 101%	46.0 ± 25 mm/ 102%	41.7 ± 22 mm	48.2 ± 20 mm/ 115%	34.3 ± 18 mm/ 82%	41.8 ± 18 mm/ 100%	46.4 ± 24 mm/ 111%
<i>Lepidium sativum</i>	58.4 ± 27 mm	32.0 ± 16 mm/ 55%	37.2 ± 35 mm/ 64%	60.3 ± 7 mm/ 103%	59.6 ± 15 mm/ 102%	49.8 ± 23 mm	48.8 ± 15 mm/ 98%	28.4 ± 13 mm/ 57%	48.8 ± 19 mm/ 98%	56.0 ± 14 mm/ 112%
<i>Fagopyrum esculentum</i>	68.4 ± 13 mm	56.6 ± 35 mm/ 83%	55.6 ± 44 mm/ 81%	70.6 ± 19 mm/ 103%	60.1 ± 29 mm/ 88%	69.6 ± 14 mm	65.9 ± 23 mm/ 95%	64.6 ± 15 mm/ 93%	73.85 ± 6 mm/ 106%	67.8 ± 15 mm/ 97%
<i>Sorghum saccharatum</i>	32.5 ± 18 mm	42.7 ± 10 mm/ 131%	29.2 ± 15 mm/ 90%	42.1 ± 15 mm/ 130%	38.3 ± 16 mm/ 118%	32.5 ± 13 mm	52 ± 16 mm/ 160%	41.6 ± 18 mm/ 128%	33.5 ± 15 mm/ 103%	39 ± 12 mm/ 120%
<i>Triticosecale</i>	46.1 ± 19 mm	28.5 ± 13 mm/ 62%	37.1 ± 16 mm/ 80%	34.0 ± 17 mm/ 74%	41.9 ± 15 mm/ 91%	38.4 ± 15 mm	41.4 ± 13 mm/ 108%	44.3 ± 7 mm/ 115%	40.85 ± 12 mm/ 106%	40.3 ± 10 mm/ 105%

solid: 723 ng L⁻¹ for the 30-day samples; liquid: 22 ng L⁻¹ and solid 332 ng L⁻¹) and apply it for 180 m³, and we consider that the solid sediment content of the slurry is 15 g L⁻¹, then we can obtain the following results for EDC (endocrine-disrupting chemical) emission: without a separator is 0.81 g/ha (810 mg/ha) and using a separator is 0.005 g/ha (5 mg/ha). Therefore, without the use of a separator, we emitted approximately 162 times more EDC into the environment. The liquid slurry was applied in the early spring period before sowing and in the summer period during row-crop cultivation and irrigation purposes.

Research shows, that approximately 90% of the oestrogen sources in the environment are derived from livestock waste. Primary sources include direct discharge, land-use of manure, and runoff containing oestrogenic compounds from CAFOs (Concentrated Animal Feeding Operations) (Liu et al. 2018). Although different oestrogens are specific to different livestock species. Cattle (*Bos taurus*) excreting more than 90% of oestrogens as 17 α -E2, 17 β -E2, and E1 as free and conjugated metabolites. However, 17 α -E2 barely discovered in the excreta of swine (*Sus scrofa*) or poultry (*Gallus domesticus*) (Hanselman et al. 2003). Wei et al. examined four oestrogens collected from 24 dairy and beef farms located in northeast China. The average concentrations of 17 α -E2, 17 β -E2 and E1 in dairy faeces were 194.6, 104.4, and 262 μ g kg⁻¹, respectively. The mean contents of 17 α -E2, 17 β -E2 and E1 in the beef waste were 104.5, 67.7 and 216.4 μ g kg⁻¹, respectively. E3 was below the detection limit in all samples (Wei et al. 2011). In the Swiss study of (Rechsteiner et al. 2020) the mean concentrations of cattle slurry were: 861 ± 367, 138 ± 126, 160 ± 205, and 397 ± 411 ng/L for 17 α -E2, 17 β -E2, E3 and E1, respectively.

Lee et al. have indicated that oestrogens from manure and cropland fertilized with animal waste pose a serious threat to the surrounding groundwater and surface waters (Lee et al. 2007).

Oestrogen contamination is a worrisome problem due to the well-known effects of oestrogen on human and animal health (Bilal et al. 2021; Bai et al. 2019; Jiang, Colazo, and Serpe 2018; Pignotti et al. 2017; Biswas et al. 2013) and on plant growth and development (Schröder et al. 2007; Janeczko and Skoczowski 2005). EDCs can be taken up by plants through active or passive processes, which mostly depend on the characteristics of the compounds and the plants (Chen et al. 2021). Based on the study by Lu et al. (2013) steroidal oestrogens were tested in fruits and vegetables obtained from local markets in Fort Pierce, FL, USA. Oestrogens were found in the vegetables (lettuce, tomato, pumpkin, potato, carrot), and in the fruits (apple, strawberry, citrus), especially in lettuce, where the 17 β -E2 was 1.26 to 3.09 μ g kg⁻¹. The toxic level for daily intake (ADI) of 17 β -E2 for a 60 kg adult is 3.0 μ g/day, according

to the FAO/WHO Expert Committee on Food Additives (JECFA) (Lu et al. 2013). Certain plants can absorb contaminants from their environment without any negative impact on their development. This mechanism is called phytoextraction. We know many plant species that have adapted to heavy metals (Abhilash et al. 2016), but unfortunately we have little information on plants that are capable of oestrogen accumulation (Chen et al. 2021; Goyal et al. 2020; Adeel et al. 2017). Continuous flow tests with wastewater show that algae and duckweed (*Lemna minor*) play a key role in oestrogen removal (Shi et al. 2010). Narrowleaf willow (*Salix exigua*) and thale cress (*Arabidopsis thaliana*) are also capable to take up oestrogens (Franks 2006). In a hydroponic study in Japan, hundreds of different plants were examined in the presence of oestrogenic substances, but only the common purslane (*Portulaca oleracea*) was the only effective phytoremediator. It removed phenol-containing chemicals, including 17β -E₂, within 24 h (Imai et al. 2007). Potato (*Solanum tuberosum*) root growth and tuber size were reduced by oestrogen (17β -E₂) (Brown 2006) while growth of maize (*Zea mays*) seedlings was inhibited by 10 mg L^{-1} concentration and the concentration of 0.1 mg L^{-1} acted as growth stimulator (Bowlin 2014). Experiments with maize (*Zea mays*) have shown that synthetic and natural oestrogens appear in the roots of maize, but only 17β -E₂ has also been detected in the stem (Card et al. 2012). In mungbean (*Vigna radiata*) (Guan and Roddick 1988) and chickpeas (*Cicer arietinum*) (Erdal and Dumlupinar 2011), E₁ and E₂ showed increased germination and vegetative growth at low ($0.1 \mu\text{M}$) concentrations, but inhibited development at high ($60 \mu\text{M}$) concentration. In the case of lentil (*Lens culinaris*), 17β -E₂ treatment resulted in increased growth

and better germination tolerance for cadmium and copper stress (Chaoui and El Ferjani 2013). It should be mentioned that xenoestrogens are also able to interfere with phytoestrogens, which regulate signal transduction between legume plants and the nitrogen-collecting *Rhizobium* bacteria living in symbiosis with the plant on their root tubers (Fox 2004). Even several researches were conducted, further ones are needed to determine whether plants used for food can uptake oestrogen from the soil through passive and active processes.

4.2 Toxicological effects

Except for the plant *Sorghum*, each seed showed approximately 25% germination inhibition in unseparated undiluted slurry. The results of the diluted samples showed that none of the dilutions gave more than 50% inhibition (10–45.2%). Considering a wastewater, this is a good outcome, but in agricultural practice, farmers aim to use slurry as irrigation water to produce larger crops without any disadvantage to the plants; moreover, the nutrient content of the slurry is supposed to stimulate plant growth. Except for *Sorghum*, there was no stimulation in the four other seeds (Figs. 3, 4).

The *Fagopyrum*, *Triticosecale*, *Lepidium* and *Sinapis* seed root lengths irrigated with the separated slurry did not show any difference from the seeds in the control plates. Only the *Sorghum* seed produced stimulation in the test, which was greater than the previous outcome (from 30 to 60%) when it was irrigated without the slurry separation technology (Figs. 3, 4). Based on the results above, we determined the no observable effect levels (NOEL) for each seed irrigated with the slurry processed using

Fig. 3 Algae cell growth (%) in different slurry dilutions in non-separated and in separated samples. Sludge is separated from the mixed liquor suspended solids in separated samples

Fig. 4 Relative germination rate of different seeds (%) in undiluted slurry in non-separated and in separated samples. Sludge is separated from the mixed liquor suspended solids in separated samples

Fig. 5 NOEL (no observed effect level) values are shown in non-separated and in separated samples, which is the highest dilution of the slurry that produces no noticeable toxic effect on tested seeds. Sludge is separated from the mixed liquor suspended solids in separated samples

separation technology, as shown in Fig. 5. *Sorghum* and *Fagopyrum* were more sensitive to the chemical composition of the slurry. Together, the results show that the algae growth inhibition test and the Phytotoxkit tests presented the efficacy of the separation technology very well. With the help of this technology, agriculture could be more productive.

Determining the amount of slurry to field application is important for nutrient management. Slurry can be beneficial from an agricultural point of view in terms of nutrient and organic matter content, but its excessive use in the

same area can be dangerous due to nitrogen (N) and phosphorus (P) contamination of groundwater (Bolan et al. 2004; Brock et al. 2006; Benke et al. 2008). According to Sterritt and Lester (1980), the agricultural use of sewage sludge can be a problem due to their heavy metal content. We know from the study by Pan et al. (2016), that if the nitrogen (ammonium, nitrite, nitrate) content is too high of the soil, it can have an inhibitory effect on plant germination. While in the experiment of Norouzi et al. (2016) tobacco (*Nicotiana tabacum* L.), alfalfa (*Medicago sativa* L.) and sorghum (*Sorghum bicolor* L.) responded well to

higher dose of nitrogen treatment (150–300 kg ha⁻¹). The slurry tested by us, as described above, did not contain toxic ammonium (ammonium-nitrogen: 0.25 g L⁻¹) and the farm under this study did not come into contact with heavy metal sources. Therefore, we attribute our toxicological results to the effect of other harmful substances in the slurry such as drugs used at the farm, detergents and natural hormonal substances in the slurry.

5 Concluding remarks

Yeast oestrogen screen shown in this study that the separation of the liquid and solid phases of slurry contributes to the reduction of oestrogenic substances. While this study is focusing on the investigation of the slurry samples of a specific year (2017) conducted on a particular cattle farm, it draws attention to the importance of the separation of the liquid and suspended solid phase of slurry before using it for agricultural purposes.

Based on the ecotoxicological studies, the growth inhibition of algae was 93%, and the inhibition of the germination of higher plant seeds was 25%, without separation. After separation of the phases, reproduction and germination were comparable with the negative control, moreover, some stimulation was detected, consequently, harmful substances (drugs, cleaning and disinfection products) were removed with the suspended solid phase.

Plant tests help with the risk management of slurry utilization, to determine the economical application of the slurry, to minimize, treat, monitor, and control the probability or impact of unfortunate events, such as bad germination and negative impact on plant growth. Moreover, this test suitable for the determination of the optimal amount of slurry utilization in order to get the optimal growth. Microbiotest applied in this study can be used effectively to the study of seed germination and can be adapted to any plant of interest.

To our best knowledge, this is the first detailed study of the liquid and solid phase of the slurry, which combines the toxicological evaluation with the measurement of oestrogenic effect of samples. Even we provide information about the oestrogen quantity and toxicity of the fractions of slurry in this study, the sorption of oestrogen compounds to the soil particles and their effects on plant growth and accumulation demands further research.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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