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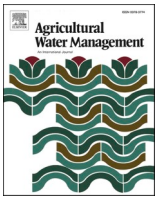

Microinjection based zebrafish embryo test for the detection of estrogenic substances in slurry based irrigation water and its combined application with yeast estrogen screen

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ABSTRACT

Waste from livestock farms, including manure, is a significant source of estrogenic pollutants in the environment. These wastes have complex matrices, necessitating the implementation of *in vivo* and *in vitro* tests in order to investigate their estrogenic effects. However, most current *in vivo* methods are limited by the toxic effect of livestock waste due to their high concentrations of organic matter. Here we propose a novel *in vivo* microinjection method which is able to avoid this limitation. In this study, the estrogen content of slurry-based irrigation water extracts from dairy cattle farms was examined using a classical *in vitro* and the newly developed *in vivo* method. The limitations of the *in vitro* system, with its absence of endogenous steroid hormone receptors and subsequent lack of elucidating complex interactions involving the estrogen receptor (ER), are complemented by the *in vivo* fish test, which allows for a more complete assessment of estrogenicity and toxicity to vertebrate animals. *In vitro* screenings were performed with the ISO 19040–1:2018 Yeast Estrogen Screen (YES). The YES test showed estrogenic activity in all 32 tested samples, which ranged from 5 to 50518 ng/L in EEQ (E2-Estradiol equivalents). The *in vivo* microinjection method was developed using a *Tg(vtg1:mCherry)* transgenic zebrafish embryo model. This model is able to eliminate secondary symptoms of hypoxia that may occur during normal aqueous exposure to high organic matter extracts. Using the microinjection method, a total of 12 samples, out of the 32 samples examined, presented no observable estrogenic effects in fish embryos based on integrated density values. In samples where the fish test showed no estrogenic effect, the liver of the larvae was significantly damaged due to sample toxicity. Our results clearly show that the combination of these methods provides a highly effective screening tool for samples containing high concentrations of organic matter.

1. Introduction

Xenoestrogens, or endocrine disrupting compounds (EDC), are widely present in our environment, and exposure to them can have serious consequences for wildlife and humans (Adeel et al., 2017; Mills and Chichester, 2005; Sumpter, 2005). Estrogenic substances have several adverse effects, such as disturbing 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 behavior (Carnevali et al., 2018; Kavlock and Ankley, 1996; Söffker and Tyler, 2012; Zhou et al., 2009).

Agricultural wastes are a source of natural and synthetic steroid estrogens as well as other estrogenic compounds. Several studies have demonstrated the presence of farm-animal-derived steroid hormones in manure and wastewater from dairy farms (Hanselman et al., 2006, 2003; Kjær et al., 2007; Lafrance and Caron, 2013). Natural steroidal estrogen hormones are excreted in the urine and feces of all species, sexes, and classes of farm animals; therefore, livestock wastes are potential sources of EDC in the environment (Adeel et al., 2017; Hanselman et al., 2006,

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2003). Agricultural manure waste is useful for agricultural landowners who use it as nutrient for plants. Manure is usually spread on soil surfaces or, more recently, injected to meet the nutrient requirements of plants as well as positively influence soil structure, humus content, water, air and heat management (Amin et al., 2014; Das et al., 2017). However, the estrogen load associated with these substances and agricultural areas may pose a risk to plants, terrestrial organisms, aquatic organisms and to humans through the food chain. Within the manure, including liquid manure (slurry), there are many types of estrogens and conjugated estrogens. In dairy cattle, E1, 17alpha-E2 and 17beta-E2 secretions have been detected in faeces and urine in their parent compound forms, as well as in their conjugated E1-sulphate and E2-alpha-glucuronide forms (Hanselman et al., 2006, 2003; Johnson et al., 2006).

The chemical diversity of these estrogenic substances makes the testing of their presence difficult, as different analytical methods are required for their detection (Sumpter, 2005). This is further complicated by the fact that these substances are mostly in the form of complex mixtures in the environment (Adeel et al., 2017; Sumpter, 2005). Effect-based screening methods offer a solution to this problem, such as the use of biomonitor/bioindicator organisms (Fetter et al., 2014; Gorelick and Halpern, 2011; Routledge and Sumpter, 1996; Sanseverino et al., 2008). Due to the diversity of estrogenic substances, and the complexity of the endocrine system, there are a lot of potential mechanisms of action, *e.g.* genomic and non-genomic pathways and autocrine/paracrine signaling networks that may be susceptible to the adverse effects of endocrine disruptors (Kiyama and Wada-Kiyama, 2015; Mueller, 2004; Zacharewski, 1998). Because of these complexities, the use of a multilevel approach for complementary *in vivo* and *in vitro* assays is essential to test the estrogenic activity of a substance or a complex sample (Beresford et al., 2000; Jarque et al., 2016; Zacharewski, 1998). Their combination and use is even recommended by OECD and the EPA as well (Huet, 2000; Fenner-Crisp et al., 2000). Additionally, the European Water Framework Directive (WFD) aims to identify potential effect-based tools (*e.g.* biomarkers and bioassays) and implement them for the monitoring and assessment of the quality of surface water bodies (Könemann et al., 2018; Wernersson et al., 2015).

Genetically modified *Saccharomyces cerevisiae* strains are *in vitro* tools that are routinely used for environmental monitoring, including measuring estrogenic effects, and they play an important role in the identification and first screening of estrogenic compounds that interact with estrogen receptors (Routledge and Sumpter, 1996; Sanseverino et al., 2008). These yeast based *in vitro* eukaryotic systems are cost-effective tools that allow for the rapid screening of a large number of compounds or mixtures in solvents (Payne et al., 2000; Viganò et al., 2008), native aqueous samples (Hettwer et al., 2018), and concentrated environmental samples (*e.g.* solid phase extracts (SPE)) (Bistan et al., 2012; Zhao et al., 2009). The YES screen has many advantages over other systems including the absence of endogenous steroid hormone receptors and a consequent lack of complex interactions between the estrogen receptor (ER) and other receptors. However, their limitations, such as being unable to model complex physiological processes in an organism (*e.g.* biotransformation, pharmacokinetics) or to detect hormone-sensitive phases of different life stages may result in unreliable predictions (Kunz et al., 2006; Zacharewski, 1998).

Among vertebrates, the role of fish in environmental risk assessment is significant, as aquatic organisms are particularly affected by EDC contamination (Mills and Chichester, 2005; Sumpter, 2005). Their endocrine system and physiological processes show strong similarities with other vertebrates, including mammals (Hill et al., 2005). Therefore, they are widely used to test for estrogenic substances and model their potential physiological effect (Bakos et al., 2019; Mills and Chichester, 2005; Sumpter, 2005).

Recently, several estrogen-sensitive biosensor lines have been created using laboratory fish models, such as zebrafish (*Danio rerio*) (Abdelmoneim et al., 2019; Bakos et al., 2019; Chen et al., 2010; Gorelick and Halpern, 2011; Tong et al., 2009). The main advantage of zebrafish as a biosensor line is the transparent body of the embryos and larvae. In our lab, we have created a novel transgenic fluorescent vitellogenin zebrafish model (*Tg(vtg1:mCherry))* which allows for the fluorescent mCherry reporter signal to be easily studied *in vivo* upon exposure to estrognic substances, without sacrificing the animal (Bakos et al., 2019; Tsang, 2010).

The testing of organic matter-rich substances in fish, especially in embryos, can cause problems due to hypoxia in the test vessel, which can lead to secondary effects that may interfere with the evaluation of the results (Küster and Altenburger, 2008; Strecker et al., 2011).

Microinjection is a simple way to introduce polar, nonpolar or organic matter-rich test substances into newly fertilized fish eggs or developing fish embryos (Colman et al., 2004; Mizell and Romig, 1997; Schubert et al., 2014; Walker et al., 1992). The results of microinjection are complex and it is not always possible to directly compare the results with classical acute and long-term tests, where embryos are exposed *via* waterborne exposure. However, microinjection is a good supplement option to conventional tests in certain cases, such as the examination of complex samples with a high organic matter concentration (Schubert et al., 2014; Csenki et al., 2019). The microinjection technique also allows for the delivery of very precise amounts of a material (Csenki et al., 2019). Shortly after microinjection, the effects on embryonic development are visible and even minor toxic effects can be distinguished from background mortality and other sublethal symptoms (Schubert et al., 2014). The distribution of the substance in the yolk is not always uniform, so it can only be sure about the absorption of the total introduced substance if the total amount of the yolk disappears from the body of the embryos. This happens at 165 ± 12 hpf (hours post-fertilization) in case of zebrafish (Litvak and Jardine, 2003).

In the case of high organic matter containing samples the method can help to eliminate the secondary effects caused by hypoxia by placing the test substance directly into the body of the embryos and not into the fluid in the test vessel, where decomposition processes can possibly begin which can reduce oxygen levels (Csenki et al., 2019). Before experiments, depending on the test endpoints and test material, several parameters of the method need to be optimized, which is a relative disadvantage compared to the commonly used aquatic exposure and standardized test protocols (Csenki et al., 2019; Schubert et al., 2014).

These study use a vitellogenin reporter transgenic zebrafish line (*Tg (vtg1:mCherry))*, whose properties must also be taken into account when designing the experiments. The zebrafish liver -where vitellogenin is also produced-, starts to develop 6 h after fertilization (6 hpf) and begins to function after 50 h (50 hpf) (Ober et al., 2003; Tao and Peng, 2009). Due to the effect of estrogenic substances, the liver is able to produce endogenous vitellogenin from the age of 2–3 days of an embryo (Chen et al., 2010). Similar and parallel/synchronous to the endogenous vitellogenin, the mCherry reporter is only expressed in the liver as well, where the first stronger fluorescent signals are detected in the embryos at 72 h of age, and then the signal strength and the size of the fluorescent area increase as the liver develops, thus facilitating the evaluation of studies (Bakos et al., 2019).

In addition to the size and maturity of the liver, the structural integrity of the liver also affects the intensity of fluorescent sign and the size of the area. The cells of the embryos, including their liver cells, can be damaged by higher concentrations of toxic substances (Bakos et al., 2013, 2019). In that case, the induction of vitellogenin production could be strongly decreased, so this had to be taken into account in the design of the experiments. Besides, embryos also have significant individual sensitivities, which must be taken into consideration too, when setting experimental numbers (Csenki et al., 2020).

Waste from livestock farms has complex matrices and poses a significant challenge to achieve accurate measurement of estrogens and their effects, however *in vivo* and *in vitro* effect directing methods are suitable to analyze the estrogenic potential of these complex samples (Noguera-Oviedo and Aga, 2016). In order to demonstrate the usefulness

of a combined *in vivo* and *in vitro* study on agricultural waste, our research was conducted at four dairy cattle farms which used a slurry management system. We focused on determining the presence of estrogenic chemicals in the slurry using two models of estrogen detection. The *in vitro* YES test (YES-Yeast Estrogen Screen), which provides a predictive value when possible human health effects need to be measured, was used to monitor the quantitative changes of estrogenic substances in slurry. The *In vivo* test utilized our estrogen-sensitive *Tg (vtg1:mCherry)* transgenic zebrafish embryos combined with a microinjection-based method in order to avoid the inherent difficulties in testing organic matter rich samples. This method enables a more complete assessment of estrogenicity and toxicity to vertebrate animals. Our aim was to develop a method that allows the transgenic fish model, when used in combination with the YES test, to detect estrogenicity in organic matter rich samples more accurately.

2. Materials and methods

2.1. Sample origin

Site 1 (K).

Location: Komárom- Esztergom County, Hungary.

The average number of animals at the site consists of: 1200 cows, including 600 milking cows, and 700 calves. The calves are housed mainly in barns with rest boxes and there are also stables with deep litter. Recreation pits are regularly cleaned by rinsing and the wastewater is drained into a slurry storage tank. This farm uses a separator in order to separate the liquid and solid parts of slurry. The solid part is treated as manure. The liquid part is stored in a pool, continuously mixed with two high-performance stirrers, and then transferred to the fields on a regular basis.

Site 2 (V).

Location: Pest County, Hungary.

The average number of animals at the site consists of: 1800 cows, including 1200 milking cows, and 1500 calves. The farm has deep-littered stables. The pre-feeding area of the stables is cleaned three times a day. The cleaned waste is drained into a storage tank, where it is stirred together with the wastewater of the milking house washing system. Since there is no separator and the dry matter content of slurry is significant, there are 4 high-performance stirrers operating continuously in order to prevent sedimentation.

Site 3 (S).

Location: Baranya County, Hungary.

The average number of animals at the site consists of: 900 cows, including 700 milking cows, and 800 calves. The farm has 50% deep-littered stables and 50% barns with rest boxes. Recreation pits are regularly cleaned by rinsing and the wastewater drained into a slurry storage tank. They use a separator machine for the separation of the liquid and solid parts of the slurry. The solid part is treated as manure. The liquid part is stored in a pool and continuously mixed with two high-performance stirrers. It is important to note that during the sampling, the separator was out of order several times, which means the samples contained increased dry matter content.

Site 4 (Sz).

Location: Somogy County, Hungary.

The average number of animals at the site consists of: 2100 cows, including 1400 milking cows, and 1700 calves. The farm has barns with rest boxes, where automated manure cleaning equipment periodically clean the stables. From the pits situated next to the barns, automated pumps transmit the slurry to the slurry storage tank, where the slurry is mixed with the wastewater of the milking house washing system. The liquid and solid part is transferred and dispensed together to the fields. Anti-inflammatory drugs, antibiotics, sex-inducing and synchronising products are used regularly. Additionally, parlour acid- and/or alkaline-based chemicals for the disinfection of milking cows and trotter care products are applied at each farm on a monthly basis.

2.2. Yeast estrogen screen for the investigation of estrogenic effect

Four samples were taken from the fresh slurry storage pools where the slurry was usually stored for 30 days. Samples were taken each time from each edge of the pools (1 L each) and then combined. In all pools, the slurry was mechanically mixed, thus the samples were considered homogenous and representative. They were collected in disposable, sterile polypropylene 50-ml Falcon tubes, which were free of DNase, RNase, endotoxins and metals. During the sampling, we were careful to avoid using other plastic accessories. Samples were placed in the refrigerator at 4 °C and processed within 4–6 days after sampling.

The samples were centrifuged in 50-ml Falcon tubes for 20 min at a speed of 3900 RCF, held at a constant temperature of 4°C. Samples, including endocrine disrupting shemicals, were extracted from the 30 ml supernatant using solid-phase micro extraction (SPE). First, the extraction cartridge (Strata X 200 mg/6 ml from Phenomenex, USA) was conditioned using a mixture of 8 ml methanol and 8 ml water at 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 a mixture of 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 μL of sample was dissolved in 1800 μL methanol, which was a 10-fold dilution. Then, further 1/50, 1/100, 1/500, 1/1000, 1/5000 and 1/10 000 dilutions were prepared for YES 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 2000 rpm velocity. The supernatant was further diluted, as in the liquid phase (1/10–1/10 000), and then used for additional testing. The estrogenic activity of the sample extracts was evaluated using the recombinant yeast strain, YES, according to the protocol ISO (1904)0–1: (2018) (ISO 19040). The YES is a reporter gene assay which can be used for the measurement of the activation of the human estrogen receptor alpha (hERα) in the presence of a sample containing compounds which cause estrogenic effects. The test strain is derived from Saccharomyces cerevisiae BJ3505 (protease deficient, MATα, PEP4::HIS3, prb-1-delta1.6 R, HIS3-delta200, lys2–801, trp1-delta101, ura3–52gal2can1). This strain harbours two plasmids. The plasmid YEPE10 contains the CUP1::hER fusion which encodes the human estrogen receptor α cloned from the MCF-7 human cell lineage under the control of the metallothionein promoter CUP1. This plasmid is selected *via* the tryptophane auxothropy of the parent strain. The second plasmid is the reporter plasmid YRPEG3 which contains the fusion gene 2ERE-CyC1::lacZ. This fusion gene express the β-galactosidase (encoded by lacZ) under the control of the iso1cytochrom c promoter from S. cerevisiae which is fused to two copies of the vitellogeninA2-gene from Xenopus laevis. This plasmid is selected *via* the uracil auxothropy of the parent strain (ISO 19040, McDonnell et al., 1991). The YES test continuously produces the human estrogen receptor. The estrogen receptor belongs to the family of nuclear hormone receptors. When the yeast encounters estrogen or a homologous molecule, it produces a β-galactosidase enzyme. β-galactosidase catalyzes the hydrolysis of the galactoside analog chlorophenol red-β-D-galactopyranoside (CPRG) and converts the yellow-orange CPRG substrate into the red chromophore chlorophenol red, yielding a dark red solution. This was measured using a microplate reader at 580 nm to determine the amount of substrate converted. The amount of estrogenic substances in the specified sample were given in relative estrogen activity or 17β -estradiol (E2) equivalents (EEQ)(ng/L) (McDonnell et al., 1991; Routledge and Sumpter, 1996; ISO 19040).

For the analysis of the estrogenic 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 to 500 ng/L) 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.

2.3. Sample preparation for microinjection

Samples were originally prepared in methanol, which caused high mortality of the zebrafish embryos in a previous study (Maes et al., 2012). 1 ml from each sample was transferred with an automatic pipette to 1.5 ml centrifuge tubes (Eppendorf AG, Germany). Methanol was removed from the tubes with a centrifugal evaporator (Eppendorf Concentrator Plus Complete; Eppendorf AG, Germany) at 60 °C for 60 min. The dry solutes were then resuspended in 500 μL DMSO (Sigma-Aldrich, Hungary) with Vortexing (VWR International Ltd., Hungary) for 20 min. Samples were stored at − 20 °C until microinjection.

2.4. Characterization of the Tg(vtg1:mCherry) biomarker zebrafish line

The zebrafish line used in these experiments is a vitellogenin reporter transgenic zebrafish line. The transgene construct used for the development of *Tg(vtg1:mCherry)* carried a long (3.4 kbp), natural vitellogenin-1 promoter sequence with a high number of ERE (estrogen responsive element) sites. The mCherry reporter is only produced in the liver, similarly to endogenous vitellogenin. The sensitivity and usability of the embryos of the line have been tested on several estrogenic compounds as well as on environmental samples (Bakos et al., 2019).

2.5. Zebrafish maintenance and egg collection

The laboratory-bred *Tg(vtg1:mCherry)* zebrafish strain was held in breeding groups of 30 females and 30 males in a Tecniplast ZebTEC recirculation system (Tecniplast S.p.a., Italy) at 25.5 ± 0.5 °C (system water: pH 7.0 ± 0.2, conductivity 550 ± 50 μS) and on a 14 h:10 h light: dark cycle. Fishes were fed twice a day with dry granulate food (Zebrafeed 400–600 μm, Sparos Lda., Portugal) supplemented with freshly hatched live *Artemia salina* twice a week. Fishes were placed in breeding tanks (Tecniplast S.p.a.) late in the afternoon during the day before the experiment and allowed to spawn by removing the dividing walls next morning. The collected eggs were incubated in system water (25.5 °C ±1 °C) in Petri dishes (diameter: 10 cm; JET Biofil, China) on 14 h:10 h light:dark cycle for 72 h. After 24 h, coagulated and/or non-fertilized eggs were sorted.

2.6. Microinjection

Three-day old hatched larvae were transferred, in groups of 60, to Petri dishes (diameter: 6 cm; JET Biofil, China). Overplus solutions were removed with a plastic pipette and were filled with 2 ml of 0.02% MS-222 (Tricane-methane-sulfonate; from Sigma- Aldrich; St. Louis, MO, US) anaesthetic solution. Anaesthetic agar plates (1.5 g agar (Sigma-Aldrich, Hungary) in 100 ml 0.02% MS-222) were prepared with specifically designed moulds for Zebrafish research (Eppendorf AG, Germany). Anaesthetized larvae were placed on the agar plate, which were then filled with anaesthetic solution. Larvae were oriented to the left side in the moulds using a cut Microloader pipette tip (Eppendorf; Hamburg, Germany). Injection needles were filled with samples, and needles were placed in the microinjection manipulator (microINJECTOR MINJ-2, TriTech Research Inc. Los Angeles, USA). The determination of the appropriate injected doses were selected with V3 liquid phase (in DMSO) samples from three sphere diameters, 50 μm (corresponded to 0.074 nL), 125 μm (1.02 nL) and 200 μm (4.17 nL). For the 3000 ng/L E2 solution used to determine the optimal treatment time and in the main test, all samples were injected with 0.074 nL dose into larvae yolk.

After the microinjection, larvae were transferred in groups of 20 in three replicates in Petri dishes (diameter: 6 cm; JET Biofil, China). The anaesthetic solution was removed and each Petri dish was filled with system water and the larvae were incubated at 25.5 °C (±1 °C) on 14 h:10 h light:dark cycle for 96 h. To determine the appropriate injected dose, the mortality of the injected larvae was checked daily for four days.

2.7. Imaging and analysis

To determine the optimal treatment time, the appearance of fluorescent signals was visually checked daily for four days in the injected larvae.

The analyses in the main test were based on the description of Csenki and colleagues (Csenki et al., 2020), as follows: System water was removed from seven-day old larvae with a plastic pipette and were filled with 2 ml of 0.02% MS-222 (Sigma- Aldrich, US) anaesthetic solution. Specially designed Petri dishes (with two cube-shaped tape, diameter: 10 cm) were filled with 4% methyl-cellulose with MS-222. Anaesthetized embryos were placed to methyl-cellulose, oriented to the left side, and pushed to the bottom of the cellulose with a cut Microloader pipette tip (Eppendorf AG, Germany). Bright field (exposure time: 6 msec, magnification: 60x), and fluorescent (mCherry filter, exposure time: 2 s, magnification: 60x) images of larvae were taken under a fluorescent stereomicroscope (Leica M205 FA fluorescent stereomicroscope, Leica DFC 7000 T camera, Leica Application Suite X, Leica Microsystems GmbH, Germany). Signals in the red range of the RGB (Red, Green, Blue) colour range was evaluated by ImageJ software (Schneider et al., 2012) based on the prepared fluorescent images. An elliptical area of the same size was selected on each image and moved to the area of the liver, then the signal strength and the size of the affected area were determined. The integrated density (ID) value is automatically calculated by the software. For the main test, ID values were determined 96 h after injection.

2.8. Statistical analyses of YES tests

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 (arigobio.com). The standard curve calibration was performed using a 4- or 5-parameter logistic regression model (Findlay and Dillard, 2007). For the determination of EEQ, the corrected absorbance of the sample extracts was interpolated in the linear range of the corresponding estradiol standard curve (Hong, 2012). The resulting EEQ concentration indicates that the estrogen activity of the sample is equivalent to the estrogen activity of an equal concentration of E2 solution.

2.9. Statistical analyses of zebrafish tests

Survival results of optimization studies are expressed as mean ± SD from three independent experiments in triplicate. Two-way ANOVA followed by Tukey's multiple comparisons test was used. Survival values of injected volumes were compared to each other at all hours. The fluorescent embryo rate in optimization studies are expressed as mean ± SD from three independent experiments, in triplicate. Kruskal-Wallis

followed by Dunn's post-hoc test was used. The number of fluorescent embryos were compared to each other.

The establishment of a reliable cut-off is of high importance to discriminate between responded and non-responded individuals. Several standard methods have been proposed to choose optimal cut-offs and all require known positive and negative tests to compute the cut-off value that will best discriminate. We adapted and applied a general formula from Classen et al. (1987), where cut-off= MEAN (mean of the integrated density values of 3 independent negative control readings) + 3 × SD (the standard deviation of independent negative control readings) (Classen et al., 1987; Lardeux et al., 2016). Thus, an integrated density value above this value was considered positive.

Results were analysed and plotted by GraphPad Prism 6.01 (GraphPad Software; San Diego, CA, US).

2.10. Animal protection

Experiments were performed in accordance with the Hungarian Animal Welfare Law (XIV-I-001/2303–4/2012) and the European directive (2010/63/EU) on the protection of animals used for scientific purposes. The experimental license numbers were: XIV-I-001/2303–4/2012 and PE/EA/349–7/2019.

3. Results and discussion

3.1. Results of the yeast estrogen screen (field analysis)

YES tests were performed to determine the estrogenicity of the samples and to gain information for developing a fish assay. Based on our test results and the description of the ISO 19040. standard, sample extracts can also be made by solid phase extraction (SPE) and show high (>90%) recovery for known (xeno)estrogens (ISO 19040). The limit of quantification (LOQ) of this method for the direct analysis of water samples is between 8 ng/l and 15 ng/l 17β-estradiol equivalents (EEQ), based on the results of the international interlaboratory trial. The upper threshold of the dynamic range for this test is between 120 ng/l and 160 ng/l 17β-estradiol equivalents (EEQ) (ISO 19040). Samples showing estrogenic potencies above this threshold were diluted for a valid quantification. To determine the accuracy and precision of the assay, 5 assay plates were individually tested with E2 standard at concentrations ranging from 2 ng/L to 500 ng/L in our laboratory. The precision was above 90% and matched the requirements of the Guidance for Industry, Bioanalytical Method Validation of 80% (CDER and CVM, 2018). The accuracy was 48.8–98.3% (2 ng/L 48.8%; 6.2 ng/L 76%; 18.6 ng/L 91.3%; 55.6 ng/L 91%; 166.5 ng/L 97.9%; 500 ng/L 98.3%) (confidence levels 95%), only concentrations between 18.6 and 500 ng/L met the CDER demands for an accuracy of ± 15% of the expected value.

During the study, 2 samples from each site were tested four times, with the liquid and solid phases remaining separate. Samples were collected from 4 dairy cattle farms with different characteristics. In general, all samples showed estrogenic activity based on the YES test. The detailed results of the yeast estrogen screen are shown in Fig. 1.

At K-farm, 5 – 15502 ng/L EEQ values were measured in the liquid phase and 70 – 2809 ng/L in the solid phase. The extremely broad range was due to the slurry separator used, since elimination of solid parts reduces the EDCs as a result of a purification process. In the period from March to July, there were outstanding values when the separator was out of order and unseparated slurry samples were tested. In the case of V-farm, 462 – 7896 ng/L estrogen activity was measured in the liquid and 252 – 6339 ng/L in the solid phases, while in the case of Sz-farm, 864 –

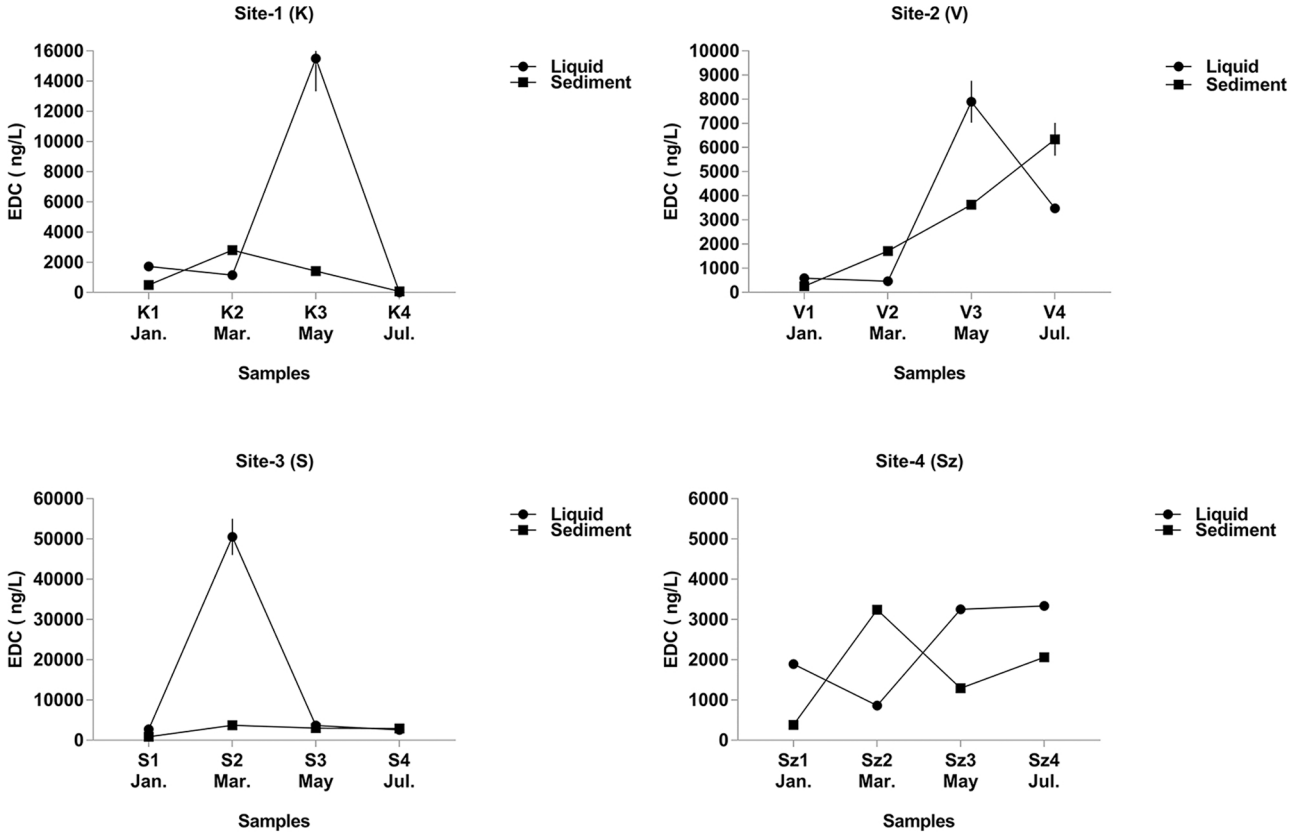

Fig. 1. Estrogen content in samples taken at different time points in E2-estradiol equivalents (EEQ) based on the YES test at four dairy cattle farms. The slurry from sites 1–4 at the dairy cattle farms have been tested by YES test 4 times a year in January, March, May and July. The estrogenic potential of the slurry is expressed as the relative potential (EEQ) to the 17β-E2 reference compound. The results show estrogenic activity in all 32 samples, which ranged from 5 to 50 518 ng/L EEQ.

3338 ng/L EEQ was measured in the liquid phase and 380 – 3242 ng/L in the solid phase. Based on the results, S-farm had the highest estrogen activities, 2583 – 50 518 ng/L in the liquid phase and 858 – 3746 ng/L in the solid phase Transgenic yeast-based assays, including the YES test, are excellent for the rapid and reliable testing of estrogenic activity in manure-based/containing samples. However, instead of direct testing of native samples, testing of different extracts is recommended in this case. The high organic matter content of native manure samples reduces the sensitivity of the YES test and may be cytotoxic to yeast which may also have a negative effect on test results (Holbrook et al., 2005).

Parent compounds (E1, 17alpha-E2 and 17beta-E2) mean the main estrogenic substances in the manure which can be detected by the YES test, the results obtained can be given as estrogen equivalent (Gadd et al., 2010b; Schoenborn et al., 2015). Significant amounts of these compounds are excreted daily by farms (300–11400 μg d-1 fecal and 500–160000 μg d-1 urinary excretion per 1000 kg live animal mass), the amount of estrogenic substances measured in manure shows high variability based in the literature (on field studies reporting the concentration of estrogens up to 16500 ng/L) (Hanselman et al., 2003; Raman et al., 2004). The actual estrogen release of a livestock farm can be affected by several factors, *e.g.* the number of pregnant animals and the stage of pregnancy, age, mass, diet, season, health status, circadian variation (Hanselman et al., 2006; Johnson et al., 2006; Schoenborn et al., 2015). Therefore, as our results show, estrogen emissions within a site are not constant or may vary from site to site.

In commercial animal production systems, faeces and urine are not collected and treated separately in most cases. However, they are often used in livestock farm's manure storage and treatment systems to control pathogens, odor, and nutrient loading to the environment (Alegbeleye and Sant'Ana, 2020; Noguera-Oviedo and Aga, 2016; Schoenborn et al., 2015). These treatments (*e.g.* solid – liquid separation, long term storage, use of lagune systems, aeration, *etc.*) have varying degrees of ability to reduce the estrogenic effect of the manure (Gadd et al., 2010a; Hutchins et al., 2007; Noguera-Oviedo and Aga, 2016; Zheng et al., 2008). On the sites examined in the study, the selected treatment procedures did not eliminate the estrogen content of the manure, however, the separator was able to significantly reduce it. The amounts of estrogenic compounds determined as estrogen equivalents in the YES test ranged from 5 ng/L to 50 518 ng/L for the four dairy farms.

The levels of estrogen detected in the YES test are substantially higher than the levels known to cause endocrine disruption in aquatic organisms, *e.g.* sex ratio skewed toward females, reversible VTG induction, reduced egg production, formation of ova-testes in males, *etc.* (Adeel et al., 2017; Brion et al., 2004; Leet et al., 2011). Our results show that the level of estrogenicity in the tested manure samples was high enough to have a measurable effect on fish, thus we began the next phase of our study using the fish embryo model.

3.2. Results of microinjection based fish embryo tests

Since this study consists of a method development and a field analysis part, it will be presented separately in this section.

3.2.1. Method development

Prior to testing, several factors had to be considered when designing the experiments. Because the primary goal in testing the samples on zebrafish embryos was to detect estrogenic activity, an experimental design had to be found where the amount of samples injected would not cause any crucial organ damage in the individuals, but contained enough estrogenic substances to induce a fluorescent signal in the liver during acute treatment.

To determine the droplet size used in the experiments, a sample (V3 liquid) that was found to be highly toxic in the yeast experiment was injected since the amount of material injected has an upper limit at which the embryos are not damaged. Furthermore, we did not have any preliminary results on the toxicity of the samples on zebrafish embryos, the samples were further concentrated to achieve the smallest possible injection droplet size. During sample concentration, the acetone originally used was replaced with DMSO because, based on literature data, more advanced embryos (from 3 dpf) appeared to tolerate this solvent better (Maes et al., 2012). These sample treatments were performed on all samples tested and were used for further experiments.

In this study, 3 different volumes of V3 sample were injected into the yolk of 3-day-old embryos, 0.074 nL (droplet diameter: 50 μm), 1.02 nL (125 μm), and 4.17 nL (200 μm). V3 marked samples were treated with three injected volumes, and then mortality was examined for 4 days. For the first two days, there was no difference in mortality results between treatments. However, 72 h after injection, the mortality data (0.074 and 1.02 nL $p < 0.05$; 0.074 and 4.17 nL $p < 0.01$; 1.02 and 4.17 nL $p < 0.01$) were significantly different. On the fourth day after injection, there was essentially no death among the smallest injected individuals. In the case of 1.02 nL treatment, only half of the embryos (51.67 ($\pm$ 7.64%)) survived, while at the highest injected dose, 28.67% ($\pm$5.51%) of the individuals were alive. No mortality occurred between control, non-injected individuals (data not shown). Based on these results, the samples were later injected with the smallest tested volume, 0.074 nL (Fig. 2A).

The length of exposure time was determined using the average amount of estrogen equivalent, calculated from the original, non-concentrated samples in the yeast test (3000 ng/L E2), which was injected into 3 day old embryos with the lowest amount (0.074 nL) as determined in the previous test. The number of individuals showing a fluorescent signal was monitored daily for 4 days after treatment.

It is clear from the results that the number of embryos exhibiting a fluorescent signal was increasing over time post injection (Fig. 2B). There were embryos as early as 24 h post treatment in which a fluorescent signal could be observed in the liver (3.33% ($\pm$ 2.89%)). A significant increase in the number of fluorescent embryos first occurred 72 h after injection. By that time, 38.33% ($\pm$ 5.77%) of the embryos produced fluorescent protein expression. On the last day of the 96-hour study period, the previous day's value doubled to 80.4% ($\pm$ 10.22%). Based on these results, this time point was chosen as the end of post-injection exposures in further experiments.

3.2.2. Field analysis

Based on the protocol established in the method development section, liquid and sediment phase slurry samples collected from 4 sites at 4 different time points were examined on transgenic zebrafish embryos according to the developed microinjection and evaluation protocol.

Regarding the tested manure samples, the number of surviving larvae was sufficient for integrated density studies. Estrogen positive samples were found for all sites both from the liquid and sediment phases based on the transgenic fish model (Fig. 3). There was a 62.5% correspondence between the yeast and fish test results, indicating estrogenic effects of the samples. Out of the 32 samples examined, a total of 12 samples did not show estrogenic effects in the fish embryo studies, based on integrated density values. From the liquid phase, only one of the K and one of the V site samples was found to be non-estrogenic (samples K1 and V3). However, K1, K3, S4, V2, V4, and SZ1 sediment phase samples were estrogenic.

Integrated density values showed high variation within each treatment, the differences in individual sensitivity mentioned above were much more significant in the case of irrigation water samples compared to individual active ingredients. Due to the large standard deviations, commonly used statistical methods did not seem to be suitable for determining estrogenicity. Therefore, data were first evaluated by the Kruskal-Wallis analysis with Dunn's multiple comparisons test (data not shown), but despite the fact that many of the treated embryos were visually observable and showed clear fluorescence in the liver, the test gave negative results in several cases. The cut-off formula (Classen et al., 1987; Lardeux et al., 2016) seemed to be much more suitable for evaluating the results, since in this case estrogenicity was in agreement with

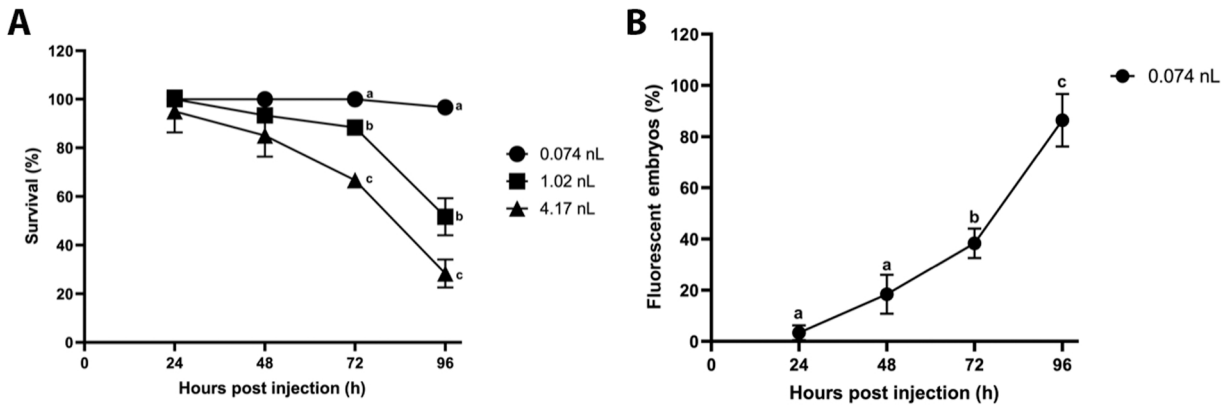

Fig. 2. Determination of optimal injected volume (A) and duration (B) of microinjection experiments Survival results of embryos injected with V3 sample are expressed as mean±SD from three independent experiments, in triplicate. Two-way ANOVA followed by Tukey's multiple comparisons test was used. Survival values of injected volume were compared to each other at all hours. At 72 h, significant differences were detected between 0.074 nL and 1.02 nL ($p < 0.05$), 0.074 nL and 4.17 nL ($p < 0.01$), 1.02 nL and 4.17 nL ($p < 0.01$). At 96 h, significant differences were observed between 0.074 nL and 1.02 nL ($p < 0.01$), 0.074 nL and 4.17 nL ($p < 0.001$), 1.02 nL and 4.17 nL ($p < 0.05$). The rate of fluorescence in the embryos injected with 0.074 nl of 3000 ng/L E2 solution are expressed as mean ± SD from three independent experiments, in triplicate. Kruskal-Wallis followed by Dunn's post-hoc test was used. Embryo rate of flourescence are compared to each other. Significant differences are indicated with different letters. Significant differences were detected between: 24 and 72 h ($p < 0.01$), 24 and 96 h ($p < 0.0001$), 48 and 72 h ($p < 0.05$), 48 and 96 h ($p < 0.0001$), 72 and 96 h ($p < 0.001$). Significant differences are indicated by different letters.

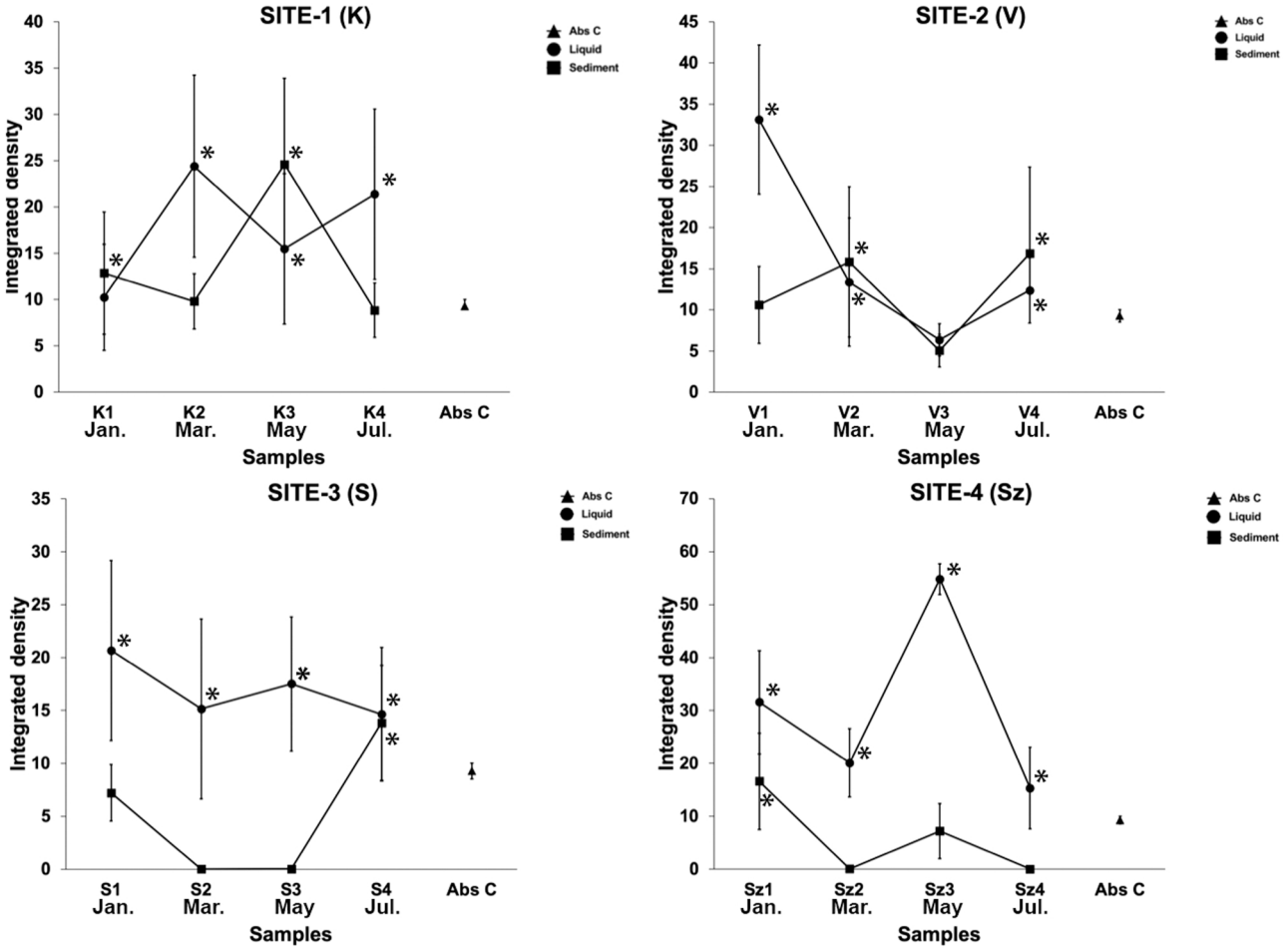

Fig. 3. Integrated density (ID) values derived from the intensity of fluorescent signals in the livers of *Tg(vtg1:mCherry)* transgenic zebrafish larvae after microinjection of samples from four dairy cattle farms. Due to the large standard deviation, the ID values were evaluated using a cut-off formula. Based on the results, out of the 32 samples examined, a total of 20 samples did show estrogenic effects in fish larvae. Estrogen positive samples are marked with an asterisk.

the visually observed fluorescence.

Examining the results further, it is apparent that for some samples, the integrated density results were of a lower value than the control integrated density results (9.274 ± 0.7403). An explicit decrease was observed only in the case of sediment samples (S2: 0.02429 ± 0.01991; S3: 0.03094 ± 0.02778; SZ2: 0.06967 ± 0.07831; SZ4: 0.06502 ± 0.08568).

Vitellogenin production in embryos was determined by developmental stage and the structural integrity of the liver (Bakos et al., 2019; Csenki et al., 2020; Faisal et al., 2020). Representative images of the livers of the embryos clearly show that, in cases where integrated density values were considerably lower, the shape and size of the liver differed from the control, suggesting that the liver is sensitive to exposure (Fig. 4). The reduced liver size could be due to liver inflammation, degeneration or necrosis, which can also be confirmed by the characteristic liver color change (He et al., 2013; Wolf and Wheeler, 2018; Zhang et al., 2016). These effects may reduce or prevent the appearance of the fluorescent signal and may indicate hepatotoxic properties of the tested sample, despite the fact that the sample may have an estrogenic effect based on the YES test, as can be seen from the results. In samples where a significant fluorescent signal was observed, the liver of the embryos did not show a considerable difference compared to the control, so it can be assumed that these samples had low toxicity to the embryos.

Numerous studies in the literature show that estrogen contamination of waterways is a concern due to the presence of these chemicals, which among other pathologies, can adversely affect the reproductive biology of aquatic wildlife, including fish (Brion et al., 2004; Hanselman et al., 2003; Leet et al., 2011; Mills and Chichester, 2005; Sumpter, 2005). The real estrogenic effect of manure released into the environment is influenced/affected by several factors, *e.g.* the relative proportion and amount of estrogenic compounds, water solubility, half-life of chemicals, biotransformation and biodegradation of microorganisms, interactions with other EDC compounds, the properties of soil and water bodies, environmental and climatic conditions, *etc.* (Adeel et al., 2017; Biswas et al., 2013; Khan and Lee, 2012; Ma et al., 2016; Yang et al., 2021;). In addition, manure also contains a number of other compounds that do not necessarily have hormonal effects, however, through their toxic effects, they may also affect vitellogenin production, as can be seen from our results.

3.3. Combined results

In summary, the microinjection method developed for *Tg(vtg1:mCherry)* embryos was shown to be suitable for studying the estrogenic effect of liquid and sediment manure samples. Extracts prepared for YES tests, after solvent exchange, were appropriate for injection experiments, so extracts were not needed to be prepared separately for the two methods. The embryo test gave a definitive yes/no answer to the estrogenic effect of the sample, however, the duration of the test was significantly longer than for the YES test. Using the two methods in combination provides a highly effective screening approach where the results of the rapid YES test are complementary to the valuable data from the new *in vivo* test method, enabling a more complete assessment of estrogenicity and toxicity.

However, results of long-term and acute toxicological tests are not comparable directly with the results of the microinjection method. The aim of this work was to develop a screening method that is able to determine if a particular sample may pose estrogenic risk when placed into the environment. This method allows for the quick and efficient detection of the estrogenic activity of organic matter rich samples, which are difficult or unable to be tested by conventional aquatic tests.

4. Conclusions

Waste from livestock farms, including manure, is one of the most significant sources of estrogenic pollutants in the environment. These wastes have complex matrices, so complementary *in vivo* and *in vitro* tests are needed in order to investigate their true estrogenic effects. Transgenic models are highly beneficial such that, in this study, an *in vivo* and an *in vitro* transgenic model were used to examine manure samples from four dairy cattle farms with different characteristics. The estrogenicity of the samples was determined by the two test methods, and the correlation of the two models was explored. The first *in vitro* screenings were performed with a classical YES test using a genetically modified *Saccharomyces cerevisiae* model. Based on the results of the YES test, a new test method was developed for a Tg(vtg1:mCherry) transgenic zebrafish embryo model, where exposure was carried out by microinjection which eliminated secondary symptoms of hypoxia that may occur during normal aqueous exposure to high organic matter extracts. The YES test results were positive for all samples tested, regardless of whether the sample came from the liquid or sediment phase. The EEQ values detected by the YES test, consistent with the literature (data), indicated orders of magnitude that could already pose a potential

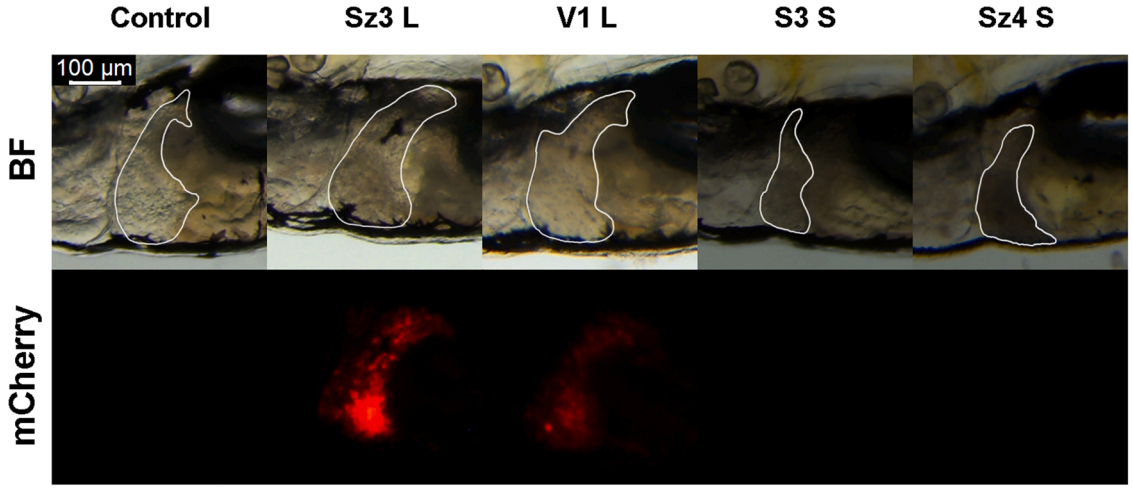

Fig. 4. Representative Bright Field (BF) and fluorescent (mCherry) images from the liver of treated *Tg(vtg1*:mCherry) zebrafish larvae. The alterations in the liver (marked with white line) size, color and shape compared to control shows, that in cases where no fluorescent activity was observed, the injected sample still caused damage to the liver.

environmental risk.

Due to the high sensitivity of the embryos, the fish test, in contrast to the YES test, was able to give primarily only yes-no answers when detecting the estrogenic effect, the overlap between the results of the two methods was 62.5% in terms of estrogen detection. However, as a complex organism, the zebrafish embryo test was also suitable for the detection of any sublethal, organ specific toxicity of samples, primarily hepatoxicity, complementing the results of the YES test. Combined, the results obtained by the YES and fish tests are more relevant from the environmental perspective as interactions between chemicals are detected and these could have a higher predictive value when possible effects need to be determined.

Several international organizations (OECD, EPA) recommend the use of complementary *in vivo* and *in vitro* methods for various toxicological/ecotoxicological tests. Using the advantages of biomarker-based monitoring and other effect-based methods is increasingly becoming the focus of research in these areas. The integration of such tools (in addition to existing methods) is also supported/promoted by the EU Water Framework Directive (WFD), as this can significantly improve its biomonitoring strategy. The inclusion of the method combination presented in the study may be an alternative to be used by the WFD for the examination of special samples with high organic matter content.

The use of these combined methods can help with the risk management of water utilization, the economical application of the water, to minimize, treat, monitor, and control the probability or impact of unfortunate events and support environmental protection. Additionally, these methods can provide assistance in the development of technologies and methods that reduce the effects of estrogenic compounds to the environment.

CRediT authorship contribution statement

Zsolt Csenki: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft. **Eduárd Gubó:** Formal analysis, Investigation, Methodology, Funding acquisition, Writing – original draft. **Edina Garai:** Formal analysis, Investigation, Methodology. **Katalin Bakos:** Formal analysis, Investigation. **Dóra Kánainé Sipos:** Formal analysis, Investigation. **Erna Vásárhelyi:** Methodology, Resources. **Béla Urbányi:** Methodology, Resources, Funding acquisition. **Pál Szakál:** Conceptualization, Methodology. **Judit Plutzer:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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